IDENTIFICATION OF NEW CITRUS NORISOPRENOIDS IN ORANGE JUICE USING TIME INTENSITY GC-O AND GC-MS

By

KANJANA MAHATTANATAWEE

A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

2004

This dissertation is dedicated to my late father.

ACKNOWLEDGMENTS

I would especially like to thank to Dr. Russell L. Rouseff, my supervisor, for his friendship and guidance throughout my graduate program. He provided me with intellectual, thoughtful discussion; encouragement; and time. Without his help and support I would not have come from Thailand and continued at the University of Florida. His high ethical standards and philosophical views will never be forgotten. I would like to thank Dr. R.M. Goodrich for all of her support, guidance and friendship. She was very kind and helpful whenever I needed her help. I also thank the other members of my supervisory committee, Dr. D.H. Powell and Dr. M.R. Marshall, Jr, for their kindness and valuable, thoughtful discussion toward my research. Especially warm thanks go to Dr. Kevin L. Goodner for his advice and support on instrumental. My special thanks go to John Smoot and Dr. Filomina Valim who assisted me any time I needed them. My appreciation goes to my dear friend Dr. Fahiem El-Borai Kora who assisted me whenever I needed his help. I extend my appreciation to all my friends at Citrus Research and Education Center (CREC) in Lake Alfred for their friendship and support. I would like also to thank my professors back in Thailand expecially Dr. Twee Hormchong, who taught me what good scientists and teachers are. My great thanks go to my beloved family. My lovely wonderful mother, father, brothers and sisters always encouraged me to follow my dream, and without their love and support, I could never be who I am today. Finally, my appreciation goes to the Siam University for giving me the financial support necessary to obtain my Ph.D from the University of Florida.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Abstract of Dissertation Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy

IDENTIFICATION OF NEW CITRUS NORISOPRENOIDS IN ORANGE JUICE USING TIME INTENSITY GC-O AND GC-MS

By

Kanjana Mahattanatawee

May 2004

Chair: Russell L. Rouseff
Major Department: Food Science and Human Nutrition

Numerous analytical studies have quantified the major volatiles in orange juice in an effort to duplicate this aroma. However, when combined, the resulting aroma does not duplicate that of orange juice, suggesting that important aroma components were missing. Citrus carotenoids have been studied primarily for their role as pigments and have generally been ignored as a source of aroma compounds. Carotenoids can be degraded into smaller (C_9-C_{13}), more volatile products called norisoprenoids. Norisoprenoids have been identified as aroma impact in foods containing carotenoids (i.e. tea, grapes, tomato, and saffron). Therefore, carotenoid-decomposition may be responsible for a portion of orange juice aroma and also aroma changes associated with thermal processing or elevated temperature storage.

Three norisoprenoids, β-cyclocitral, β-damascenone, α-ionone, were fully identified in fresh and pasteurized orange juice for the first time. Beta-ionone was also detected but had been previously identified. Identification was based upon SPME

headspace, GC-O, and GC-MS data. Only two norisoprenoids (β-damascenone and β-ionone) were detected in reconstituted juice that had been thermally concentrated. Peel oil from the same fruit contained only β-damascenone and β–cyclocitral.

Concentrations of β-cyclocitral, β-damascenone, α-ionone, and β-ionone were determined using standard addition SPME and GC-MS and found to be 145, 0.09, 47 and 83 μg/L respectively. The concentration of β-damascenone increased from 0.09 to 0.85 μg/L after thermal concentration and reconstitution. Orange juice norisoprenoids contribute approximately 8-10% of total aroma intensity as determined from combined aromagram peak heights and 60-80% of the total floral-category.

Known norisoprenoids precursors (β-carotene, α-carotene, α-cryptoxanthin, β-cryptoxanthin, and neoxanthin) were identified in Valencia orange juice using C30 reverse phase HPLC with photodiode array detection.

Thermal decomposition products of β-carotene in citric acid solutions buffered at pH 3.8 were examined during 35°C storage using GC-O and GC-MS. Beta-cyclocitral, β-homocyclocitral, β-damascone and β-ionone were detected after 2 weeks thus demonstrating that β-carotene can produce norisoprenoids. Since half of the α-carotene, α-cryptoxanthin, β-cryptoxanthin structures share the identical structure as β-carotene, these carotenoids must be considered potential norisoprenoid sources as well.

CHAPTER 1
INTRODUCTION

The delicate aroma of fresh orange juice is the result of a complex mixture of volatiles blended in specific proportions. Numerous analytical studies *(1-5)* have identified and quantified the major volatiles in orange juice in an effort to duplicate this aroma. However, when combined, the identified volatiles could not duplicate orange juice aroma, suggesting that important aroma components were missing. Early orange juice gaschromatography olfactometry (GC-O) studies *(4, 6, 7)* have shown that many of the aroma-active compounds in orange juice exist as low-level volatiles that are difficult to detect using typical flame ionization detector (FID) or mass spectrometer (MS) detectors. Furthermore, these studies demonstrated that the major volatiles in orange juice have little to no aroma activity. Recent orange juice GC-O studies *(5)* quantified the 25 most intense aroma-active compounds in fresh juice, using isotope dilution analysis. Model solutions of the aroma components in orange juice based on GC-O studies have come closer to duplicating the aroma of fresh orange juice than model systems based on the composition of the volatiles found in highest concentration.

Carotenoids are too large (C_{40}) to be volatile under normal conditions. Because they contain a highly conjugated double bond structure, they can be degraded by enzyme, chemical, and/or thermal reactions to form a wide range of structures, depending on which double bond is broken. Some of their smaller (C_9-C_{13}), volatile, decomposition products are called norisoprenoids. Norisoprenoids have been shown to have significant aroma impact in fruits, vegetables and spices such as grapes, apples, lychee, starfruit,

mango, tomato, saffron, cured tobacco, and black tea *(8-16)*. Only a single norisoprenoid, β-ionone, has been identified to date in fresh orange juice *(4, 5)*

More than 50 carotenoids have been separated and identified from the juice of three varieties of *Citrus sinensis* (Shamouti, Valencia, and Washington Navel) using column chromatography combined with thin layer chromatography (TLC) *(17)*. Some of these carotenoids (such as β-carotene, α-carotene, neoxanthin, β-crytoxanthin, lutein, violaxanthin, and canthaxanthine) have the structural potential to form potent norisoprenoid fragments *(18-22)*. Furthermore, β-carotene in tomato products has been shown to produce β-ionone and β-cyclocitral (*23*). Beta-ionone and α-ionone have been generated from β-carotene and α-carotene respectively in carrots (*24*). Neoxanthin in grapes has been shown to be a source of β-damascenone (*25*). Prior orange juice carotenoid studies were primarily directed toward the contribution of carotenoids to juice color and for vitamin A content. They have been generally ignored as precursors of aroma compounds. Since orange juice has so many carotenoids that could serve as precursors for a wide range of norisopernoids, the objectives of this research were to:

1. Confirm the presence of possible carotenoid norisoprenoid precursors in orange juice using HPLC and photodiode array detection. (Chapter 3)
2. Determine if additional norisoprenoid are present in orange juice. Characterize and identify these new norisoprenoids. (Chapter 4)
3. Determine the relative aroma impact of carotenoid degradation products (norisoprenoids) to the total aroma impact of orange juice in fresh, pasteurized, and reconstituted from concentrate juice. (Chapter 5)
4. Develop quantitative procedures to isolate and quantify orange juice norisoprenoids using static headspace SPME with GC-MS. (Chapter 5)
5. Determine if β-carotene can form norisoprenoid degradation products at 35°C storage in model solutions. (Chapter 6).

CHAPTER 2
LITERATURE REVIEW

Orange Juice Aroma

The aroma of fresh orange juice is composed of a complex mixture of aldehydes, esters, ketones, alcohols and terpenes blended in specific proportions. Numerous studies (*1-5*) aimed at identifying the flavor volatiles in orange juice have led to the identification of about 200 volatiles, but no single aroma character impact for orange flavor has ever been reported. GC-olfactometry and orange juice volatile quantification have been used to gain a more accurate understanding of their contribution to orange flavor (*2, 5-7*). Early orange juice GC-O studies have demonstrated that the orange juice volatiles present in highest concentration have little to no aroma activity and many aroma active compounds exist as low-level volatiles that are difficult to detect using typical instrumental detectors.

Carotenoids

Carotenoids are primarily responsible for the colors of many plants, birds, and insects; but also serve as plant photoprotection agents during photosynthesis, and as essential human nutrients. However, the least-appreciated role of carotenoids is their function as aroma precursors.

Carotenoids are tetraterpenes (C_{40}) resulting from the joining together of eight molecules of isoprene (C_5) through "tail-to-tail" condensation. Most carotenoids have a C_{40} carbon skeleton. The ends may or may not be cyclized into six membered rings. If the ends are not cyclized, the molecule is termed acyclic. There are two main groups of carotenoids:

the hydrocarbon group, which contain only carbon and hydrogen; and the xanthophyll group, which contain carbon, hydrogen, and oxygen. Oxygen in xanthophylls is usually found as either hydroxyl-(monols, diols and polyols), epoxy- (5,6 and 5,8-epoxides), methoxy, aldehyde, oxo, carboxy and/or esters. Hydroxyl substitution primarily occurs at the C_3 position in the ionone ring; and a carbonyl substitution usually occurs at the C_4 position in the β-ionone ring. In most of the cyclic carotenoids, the 5,6- and 5',6'-double bonds are the most susceptible to epoxidation. The unconjugated double bond in the α-ionone ε ring does not undergo epoxidation. Allenic carotenoids have a C=C=C grouping at one end of the central chain, and acetylenic carotenoids have a –C≡C– bond in position 7,8 and/or 7',8' (*26, 27*). Figure 2-1 shows acyclic carotene (lycopene), bicyclic carotene (β-carotene), the monol β-cryptoxanthin, and the diol zeaxanthin.

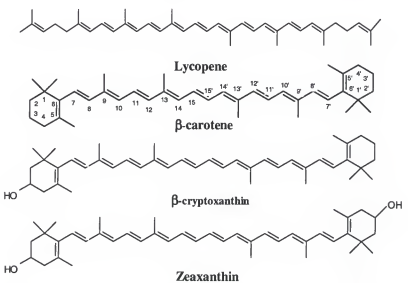

Figure 2-1. Examples of carotene and xanthophyll carotenoid structures.

Norisoprenoids

Norisoprenoids are volatile C_9-C_{13} fragments from the degradation of the C_{40} carotenoids. The formation of norisoprenoids from carotenoids is thought to proceed via

enzymatic and nonenzymatic pathways. Nonenzymatic cleavage reactions include photo-oxygenation (*18*), auto-oxidation (*28, 29*), and the thermal degradation processes (*30, 31*). The *in vivo* cleavage of the carotenoid chain is generally considered to be catalyzed by dioxygenase (lipoxidase and peroxidase) systems and require molecular oxygen and other cofactors for activity. The polyene chain of carotenes is readily oxidized, giving rise to cyclic and acyclic compounds (often having an oxygen-containing functional group on a trimethylcyclohexane ring, or an oxygen-containing functional group on the allyllic side chain). Although all the in-chain double bonds seem to be vulnerable to enzymatic attack, in actuality the formation of major fragment classes with 10, 13, 15 or 20 carbon atoms are most common (see Fig. 2-2). In fruit tissues, the bio-oxidative cleavage of the 9,10 (9',10') double bond seems to be the most preferred (*15, 32, 33*).

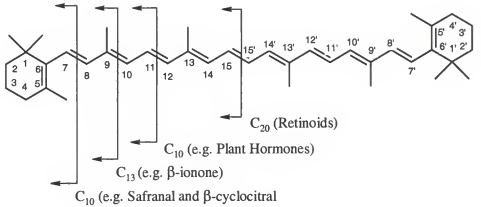

Figure 2-2. Major fragment classes of carotenoid biodegradation.

Norisoprenoids Formation from Carotenoids

Norisoprenoids can be generated from carotenoids via either direct cleavage or cleavage followed by subsequent reactions. In the latter process, three steps are required to generate an aroma compound from the parent carotenoid: 1) the initial dioxygenase cleavage, 2) subsequent enzymatic transformations of the initial cleavage product giving

rise to polar intermediates (aroma precursors), and 3) acid-catalyzed conversions of the nonvolatile precursors into the aroma active form (*32*). One example illustrating these reaction is the formation of β-damascenone from neoxanthin (Fig. 2-3). The primary oxidative cleavage product of neoxanthin, grasshopper ketone, must be enzymatically reduced before finally being acid-catalyzed converted into the odoriferous ketone. In the direct process, the target compound is immediately obtained after the initial cleavage (i.e., formation of α- and β-ionone directly from α- and β- carotene) (*34*).

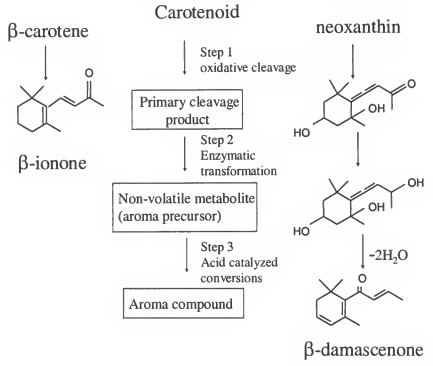

Figure 2-3. General steps for the conversion of carotenoids into flavor compounds, showing the formation of β-ionone and β-damascenone from β-carotene and neoxanthin respectively (Winterhalter, P., Rouseff, R. Carotenoids-Derived Aroma Compounds: An Introduction. In Carotenoid-derived Aroma Compounds; P. Winterhalter and R. Rouseff, Eds.; American Chemical Society: Washington, DC, 2002, Fig. 4, page 12).

Recent studies have shown that some of the volatile C_{13}-compounds are not free, uncomplexed plant constituents; but rather are derived from less or nonvolatile precursors such as polyols, glycosides, and glucose esters. Carotenoid degradation is initiated by

oxidative cleavage of the intact carotenoid. After further enzymatic transformation steps, the primary cleavage products are converted into reactive C_{10} to C_{13} fragments of the initial carotenoid. These volatile fragments can be stabilized and made nonvolatile by glycosylation (which involves glycosyltransferases of those norisoprenoid compounds possessing a hydroxyl group) (*35, 36*).

Glycosilation stabilizes and solubilizes norisoprenoids in plant systems. Degradation of the glycoconjugates librates the potent volatile and can produce profound aroma changes. This process can be acid-catalyzed (e.g., during fruit processing) (*9*) or enzymatic (e.g., during fermentation) (*37*). Another important class of precursors is the polyols, which upon (allylic) elimination of water is transformed into volatile forms. An example is the reactive allyl-1,6-diol that, under gentle reaction conditions (natural pH, room temperature), is converted into isomeric theaspiranes (*38*). A third class of carotenoid-derived aroma precursor is glucose esters (e.g., C_{10}-compounds derived from the central part of the carotenoid chain, which is left after the cleavage of the endgroups) that gives rise to isomeric marmelo lactones, key aroma constituents of quince fruit (see Fig. 2-4) (*39*).

Norisoprenoids have been shown to have significant aroma impact in fruits (apple, mango, grape, starfruit, lychee, passion fruit, nectarine, etc.) (*9-12, 19, 36, 40*) vegetables (tomato (*41*)), spices (saffron (*14*) and paprika (*42*)), leaf products (tobacco (*43*) and tea (*44*)) as well as roses (*45*), wine (*46*) and oak wood (*47*).

Apple

Beta-Damascenone is a potent aroma compound found in a variety of natural products, with a threshold of 0.002 μg/L in water (*48*). Eight separate β-damascenone

precursors have been detected in apples (*Malus domestica* Borkh. cv. Empire) (*49*). The most abundant precursor, present at 4.6 ng/g, was the 9 (or 3) -α-L-arabinofuranosyl-(1,6)-β-D-glucopyranoside of the acetylenic diol. The second most abundant precursor, present at 3.1 ng/g, is a more polar glycoside of the acetylenic diol. (*49*). Beta-Damascenone contributed 32% of the total aroma potency of heated apple juice, but only 1.6% of the total aroma of fresh apple juice as determined by GC-O. Thus, most of the β-damascenone in heated apple juice was generated from nonvolatile precursors during thermal processing (*9*).

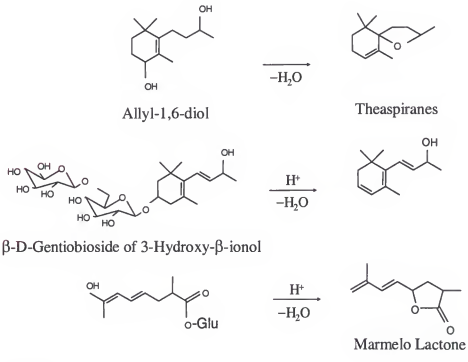

Figure 2-4. Formation of norisoprenoids aroma compounds from different classes of precursors (i.e., polyols, glycosides, and glucose esters).

Tomato

One of the most marked differences between the fresh tomato and the paste is the almost complete loss of the major contributor to fresh tomato aroma, (Z)-3-hexenal. The most notable increase is with the potent odorant, β-damascenone, which shows a 10 fold

increase in concentration in the paste (*50*). Beta-ionone in tomatoes seems to be formed mainly by an oxidative mechanism. It was not detected among the glycoside hydrolysis products. The compound β-damascenone was shown to be produced in fruits from hydrolysis of glycosides via an intermediate acetylenic compound megastigm-5-en-7-yne-3,9-diol. This appears to be the final step in tomato volatile norisoprenoid formation (*51*). Three experimental lines of tomato: a high-β-carotene line; a high-lycopene line; and a low-carotenoid line were examined for their norisoprenoid content. In fresh tomato, the high β-carotene line produced the highest concentrations of β-ionone (17 μg/L, versus 1 μg/L in the low-carotenoid line) and β-cyclociral produced 30 μg/L in the high carotene line versus 0 μg/L in the low-carotene line). Both norisoprenoids are known biological or chemical degradation products of β-carotene. The high lycopene line, however, did not show any significantly higher concentration of the expected lycopene degradation products, 6-methyl-5-hepten-2-one, 6-methyl-5-hepten-2-ol, or geranylacetone. It did show a significantly higher value for geranial (21 μg/L) compared to that of the common line (12 μg/L). Geranial could be considered a lycopene-degradation product (*41*).

Saffron

Safranal (monoterpene aldehyde, $C_{10}H_{14}O$) is the characteristic impact compound of saffron (dried stigmas of *Crocus sativus*), formed in saffron during drying and storage by hydrolysis of picrocrocin. Picrocrocin was the colorless glycoside of the aglycone, 4-hydroxy-2,6,6-trimethyl-1-carboxaldehyde-1-cyclohexene (HTCC), which was the main substance responsible for the bitter taste of saffron. Safranal was not present in the fresh stigma. Its concentration in saffron depended strongly on both the drying and

storage conditions. Additional flavor compounds in saffron were formed upon cooking of the spice (*52*). Aroma isolates of saffron have been prepared by simultaneous distillation extraction (SDE) at pH 2.6 as well as liquid-liquid extraction using pentane: diethyl ether (1:1) as solvent. Aroma activity and relative aroma strength was determined using aroma extract dilution analysis (AEDA). Compounds with high FD-factors were safranal and 2-hydroxy-4,4,6-trimethyl-2,5-cyclohexadien-1-one as well as linalool and isophorone. The 2-hydroxy-4,4,6-trimethyl-2,5-cyclohexadien-1-one was only detected in the SDE isolate and not in the liquid-liquid extract. This result shown the presence of certain forms of precursors, which upon heat treatment are converted into the aroma compound 2-hydroxy-4,4,6-trimethyl-2,5-cyclohexadien-1-one (*53*).

Grape and Wine

Norisoprenoids are important aroma constituents of grape and wine. They are thought to arise from carotenoid breakdown; and occur in grapes as glycosidically bound precursors. The major carotenoids in grape are β-carotene and lutein, representing nearly 85% of the total carotenoids. These are accompanied by minor carotenoids such as neoxanthin, violaxanthin, lutein-5,6-epoxide, zeaxanthin, neochrome, flavoxanthin, and luteoxanthin (*54*). Grape carotenoids decrease progressively during maturation, with a concomitant increase of the volatile compounds. This degradation would occur during berry metabolism either enzymatically or by chemical pathway in acid medium (*54, 55*). This would account for the presence of volatile compounds, such as β-ionone and β–damascenone, identified in grape (*56*) and possibly originating in carotenoids (*36*). Many norisoprenoids occur in grapes as glycosidic precursors. Enzymatic and acid

cleavage during crushing, fermentation, and bottle-aging result in cleavage of the bound sugar moiety releasing the free norisoprenoid aglycone (*57, 58*).

Gas Chromatography-Olfactometry

Gas chromatography-olfactometry (GC-O) is a technique that allows the effluent from the GC column to be evaluated for aroma activity using the human nose. The effluent from the GC column is usually split between an FID detector and sniff port. The human being detects which of the volatiles eluting from a GC column are aroma active, as well as to describe aroma quality, and to estimate aroma intensity. The FID detector is used as a general mass detector. Some of the GC-O techniques available are Charm® Analysis (*59*), Aroma Extraction Dilution Analysis (AEDA) (*60*), and OSME (*61*) which is a time intensity method. Charm® Analysis and ADEA are based on the determination of odor detection thresholds of the compounds through a series of dilutions. Both define aroma strength in terms of its dilution strength. OSME determines intensities based upon magnitude estimation using a variable potentiometer to estimate intensity. Da Silva et al. (*61*) suggested that dilution techniques might not give accurate values of aroma intensity, since the odorants may have different dose-response functions above their thresholds. Steven's law (*62*) establishes that the odor intensity (I) of a compound increases as a power function (n, which varies from compound to compound) of the concentration within a certain range of concentration (C) directly above the detection threshold (T). The law is commonly expressed as:

$$I = k\,(C\text{-}T)^n,$$

where k represents the proportionality constant. Response will increase once the threshold concentration is exceeded. Even though not defined by the above equation, a limit will be reached where the sensory response will no longer increase with increasing

concentration. This point is defined as saturation. When a sample is diluted below the odor-detection threshold, there will be no sensory response. Steven's law suggests that two different compounds (A and B) at the same concentration, with similar detection thresholds but with different exponents (*n* values), will produce different dose-odor intensity profiles (Fig. 2-5). Individual odors will contribute differently to the overall food aroma intensity, depending on their concentration and *n* value (*61*).

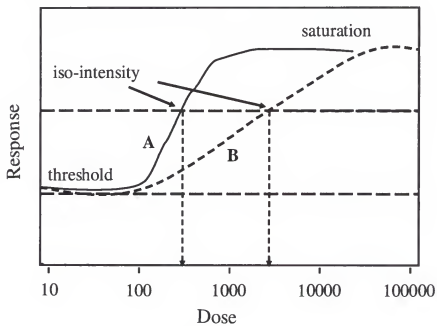

Figure 2-5. Steven's law, comparing two difference compounds: A= compound A, B= compound B.

The OSME is a time intensity procedure that determines the intensity of the perceived odor without dilution. In this method, the trained assessors sniff the effluents from GC mixed with humidified air, and directly record the odor intensity and duration of each odor active component while describing its odor quality. Intensities of individual components are plotted versus elution time; and the resultant graph is known as an aromagram.

Solid Phase Microextraction

Solid phase microextraction (SPME) is a relative new technique whereby analytes of interest partition from the sample matrix into a polymeric coated silica fiber, developed by Pawliszyn and co-workers (*63*). It is a simple, rapid, solventless technique to sample static headspace volatiles. A 1 or 2 cm length of fused silica fiber, coated with a polymer, is bonded to a stainless steel plunger and installed in a holder that looks like a modified microliter syringe. The plunger moves the fused silica fiber into and out of a hollow needle. To use the unit, the analyst draws the fiber into the needle, passes the needle through the septum that seals the sample vial, and depresses the plunger, exposing the fiber to the sample or the headspace above the sample. Organic analytes adsorb to the coating on the fiber. After adsorption equilibrium is attained, the fiber is drawn into the needle, and the needle is withdrawn from the sample vial. Finally, the needle is introduced into the gas chromatographic (GC) injector, where the adsorbed analytes are thermally desorbed and delivered to the GC column.

The application of headspace SPME to flavor volatile compounds has been employed in the study of flavor volatiles in orange juice using a PDMS coated fiber (*64*), a Carboxen-PDMS fiber (*6*), a DVB/Carboxen/PDMS fiber (*65*), PDMS and polyacrylate fiber (*66*). The partition coefficients of the polymeric coatings for the analyses differed markedly. For example, terpenes such as α-pinene, β-myrcene, γ-terpinenes, and limonene are all nonpolar, and were extracted to a higher degree into the nonpolar PDMS coating (*66*). Corresponding PDMS extracted the least amount of the more highly polar volatiles, PDMS/DVB and Carbowax/DVB had partition coefficients higher than that of PDMS for the most polar molecules (*67*). The Carboxen-PDMS fiber coating was more

selective for terpenes than early eluting alcohols and aldehydes (*6*). Polyacrylate was more effective in extracting highly polar compounds such as methanol and ethanol (*66*).

Orange Juice Norisoprenoids

Only a single norisoprenoid (β-ionone) has been reported and completely identified in fresh orange juice (*4, 5*). Recently, β-damescenone has been reported in heated orange juice, but not completely identified (*65*). With so many carotenoid precursors present in orange juice, it seems highly likely that additional norisoprenoids would also be present. The primary objective of this study was to determine if these additional norisoprenoids were present in orange.

CHAPTER 3
HPLC DETERMINATION OF CAROTENOID NORISOPRENOID PRECURSORS IN ORANGE JUICE

Introduction

The color of orange juice is due to a complex mixture of plant pigments called carotenoids. Over 50 carotenoids have been identified in orange juice including β-carotene, α-carotene, β-cryptoxanthin and neoxanthin (*17, 68*). In addition to acting as plant pigments and free radical scavengers (produced during photosynthesis), these large highly conjugated molecules can break down forming smaller, highly potent aroma volatiles called norisoprenoid (*15, 39, 69, 70*). The structure of β-carotene is shown below. If this molecule hydrolyzes between carbon atoms 9 and 10, a C_{13} norisoprenoid, β-ionone is formed. If the molecule hydrolyzes between carbon atoms 7 and 8, then a C_{10} norisoprenoid, β-cyclocitral is formed.

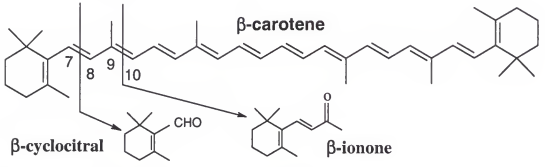

Figure 3-1. Possible degradation pathways for the formation of β-cyclocitral and β-ionone from β-carotene.

Beta-cyclocitral, β-damascenone, α-ionone, and β-ionone are some of the volatiles reported in tomato (*23*), wine (*71*), tobacco (*22*) and tea (*16*) as aroma active compounds.

In each case carotenoids have been shown to be their precursors. Citrus carotenoids have been examined using a variety of chromatographic techniques such as column and thin layer chromatography, TLC, and more recently high performance liquid chromatography, HPLC (*68, 72, 73*). Once separated, individual carotenoids have been identified primarily by their unique "three fingered" visible absorbance patterns. In orange juice, most oxygen containing carotenoids are esterified with C12-C18 fatty acids (*74*) thus increasing their size and structural complexity. The most common practice is to de-esterify (hydrolyze) these esters so that each carotenoid will elute as a single peak rather than several smaller peaks with various fatty acids attached. However, even with hydrolysis, the large numbers and subtle structural differences of orange juice carotenoids provide a severe separation challenge. To complicate matters further, carotenoids are sensitive to heat, light and oxygen, thus artifacts are readily formed during sample preparation and/or analysis steps. HPLC equipped with a photodiode array detector is the preferred analytical technique of choice to separate and quantify carotenoids without artifact formation. Both normal phase and reverse phase chromatography have been employed to separate these plant pigments, but the reverse phase approach offer the most advantages. The most common reverse phase column is C-18 and most of the early carotenoid separations employed this column. However, in recent years a C-30 reverse phase column has been developed especially for carotenoid separations. Several investigators (*68, 75, 76*) have employed this column with ternary solvent gradient and photodiode array detector to isolate and identify the complex mixture of carotenoids in orange juice.

Objectives

The objective of this study was to confirm the presence of specific carotenoids in Valencia orange juice which could serve as norisoprenoid precursors. The specific carotenoids of interest include: α-cryptoxanthin, β–cryptoxanthin, α-carotene, β-carotene and neoxanthin because they possess the structural features needed to serve as precursors to the newly identified norisoprenoids. (See Objective #1)

Materials and Methods

Carotenoid Extraction

The carotenoid extraction method according to Lee et al. (*75*) was carried out with slight modification. A 25 mL aliquot of Valencia juice was extracted with 50 mL of a mixed solvent (hexane:acetone:ethanol, 50:25:25) using a Omni mixer homogenizer (model no. 700, Lourdes, Vernitron Medical Products, Inc. Carlstadt, NJ). It was extracted for 5 min at medium speed in ice bath, and centrifuged (CR412, Jouan, Inc., Winchester, VA) for 10 min at 4000 rpm and 10°C. The top layer of hexane containing pigments was collected and concentrated to dryness in rotary evaporator.

Carotenoid Saponification

Saponification was carried out according to Noga and Lenz (*77*) with slight modification. The dried pigment was redissolved with 2 mL of methyl tert.-butyl ether (MTBE), and placed in a 40 mL vial. Two mL of 10% methanolic potassium hydroxide (KOH) was added to the sample and the headspace was blanketed with nitrogen before closing. The sample was wrapped with aluminum foil to protect it from light, and placed at room temperature for 1 hour. The sample was then transferred to separatory funnel to which 5 mL of water was added and 2 mL of 0.1% butylated hydroxyl toluene (BHT) in MTBE, and the aqueous layer removed. Additional water rinses were carried out until

free of alkali. The MTBE layer was then filtered through a small glass column filled with deactivated glass wool (Restek Corporation, PA) and anhydrous sodium sulfate (Fisher Scientific, NJ) to remove residue water from MTBE layer. Each sample was concentrated by evaporation with nitrogen, and the volume adjusted with 0.1% BHT in MTBE to 1 mL and placed in sealed amber vials under refrigeration (4°C) until analyzed.

HPLC Procedure

Carotenoid pigments were analyzed according to Rouseff et al.(*68*) by reverse phase HPLC using ternary gradient of water, methanol, and MTBE with photo diode array detection (PDA] by reverse phase HPLC using ternary gradient of water, methanol (MeOH), and MTBE with photodiode array detection (PDA). The 4.6 mm i.d. x 250 mm YMC Carotenoid™ 5 µm column (YMC, Inc., Waters Corporation, MA) was used. The chromatographic system consisted of autosampler, LC pump, and PDA detector (Surveyor, ThermoFinnigan, CA). The PDA was set to scan from 280 to 550 nm. Three separate data channel were set to record the absorbances at 350, 430, and 486 nm with spectral bandwidths of 1 nm. Data were collected, stored, and integrated, using the Atlas software (Atlas 2003, Thermo Electron Corporation, Cheshire, UK). All reagents used were HPLC grade (Fisher Scientific, NJ). One standard, β-carotene, was purchased from Acros (Acros, NJ). The initial ternary gradient composition consisted of 90% MeOH, 5% water, and 5% MTBE. The solvent composition changed in a linear fashion to 95% MeOH and 5% MTBE at 12 min. After the next 8 min (at 20 min) the solvent composition was 86% MeOH and 14% MTBE. At this composition the solvent composition was gradually changed to 75% MeOH and 25% MTBE at 30 min. The final composition was 50% MeOH and 50% MTBE at 50 min. Intial conditions were

reestablished within 2 min and reequilibrated for 15 min before the next injection. Flow rate was 1 mL/min and injection volume was 10 μL.

Results and Discussion

Carotenoids of Interest

Although over 50 carotenoids have been identified in orange juice, only a few possess the structural requirements to produce potent norisoprenoids. The structures of the carotenoids which have been shown to produce norisoprenoids of interests in other food systems (*15, 23, 31, 39, 69, 70, 78*) are shown in Fig. 3-2. Hydrolysis points are indicated with arrows and resulting norisoprenoid indicated as text.

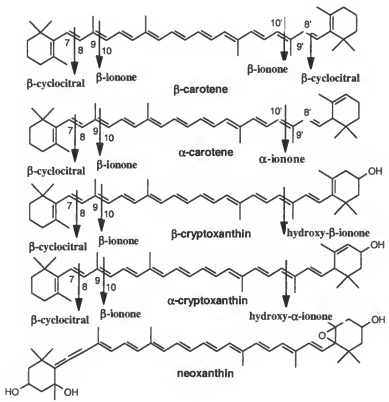

Figure 3-2. Carotenoid precursors of selected norisoprenoids including neoxanthin, the indirect precursor of β-damascenone.

It is worth noting that the structures of the left half of the first four carotenoids are identical. Each of these four carotenoids can produce either β-cyclocitral or β-ionone.

The right half of these four carotenoids differ considerably. The right half of β-carotene can produce both β-cyclocitral and β-ionone because it is identical to the left half of the molecule. The right half of α-carotene can produce α-ionone and neither α- or β-cryptoxanthin produce norisoprenoids which were observed in orange juice. The final carotenoid of interest, neoxanthin, has been shown to produce β-damescenone in a three step process (*34*).

Hydrolysis Conditions

As previously discussed, citrus carotenoids must be hydrolyzed to simplify the separation due to the complexity from the multiple natural esters formed from C_{12}-C_{18} saturated fatty acids (*74*). Hydrolysis conditions must be optimized in order to free the esterified carotenoids into a single form but not so long as to promote alkaline hydrolysis of the carotenoids. Concentration of alkali, reaction time and temperature are the variables of interest. In recent years, most carotenoid studies have employed 0.1 M KOH and room temperature so only reaction time was optimized for this study. Chromatograms with no saponification showed 80% of the total peak area eluting as an unresolved band of peaks during the last quarter of the chromatogram. As saponification time increased, the number of peaks at the end of the chromatogram diminished and the peaks were more evenly distributed during the chromatographic run. Saponification times in excess of one hour did not reduce the number of late eluting peaks and total carotenoid peak area was lower at 4 hours and overnight saponification compared to the one hour saponification. Therefore the one hour saponification was used for the remainder of the study.

HPLC Separation

C-30 carotenoid columns with ternary gradient solvent systems and photodiode array detectors have been employed to separate and identify carotenoids in citrus (*68, 75, 76*). In this study saponified carotenoids were separated using a C-30 carotenoid column with ternary solvent gradient system of water, methanol, and MTBE with photodiode array detection. The resulting separation is shown in Figure 3-3. More than twenty-four carotenoids were separated as distinct peaks and sixteen of these peaks were identified based on their spectral characteristics (Table 3-1), relative elution order compared to literature values and authentic standards. As seen from the chromatogram in Fig. 3-3, peaks 11 and 20 corresponding to cis-violaxanthin and β-cryptoxanthin (15.76 and 12.34 percentage of total peak area, respectively). They have been reported as the major carotenoids in earlier studies. Beta-cryptoxanthin is well accepted as the major contributor to the orange color of the juice (*79*) because of its relatively high concentration an overall absorbance in the red/orange range of the spectrum. The last four peaks (21-24) are due to a variety of carotenes which are not completely resolved. Both α- and β-carotene are of particular interest in this study because of their ability to produce a range of norisoprenoids which have been observed in other food products. In addition, peaks 18 and 20 were well resolved and corresponded α- and β-cryptoxanthin from the match of retention times and spectral characteristics compared to authentic standards. The final peak of interest was neoxanthin and this compound corresponds to peak 4 which is neither particularly well resolved nor large.

The large number and similarity of orange juice carotenoids make separation difficult. Thus even under the best chromatographic systems, some peaks will not be

well resolved (i.e., peaks 8-10) and make accurate identification difficult. Lutein and zeaxanthin (peaks 14 and 15, respectively) are usually difficult to resolve as they differ only in the position of a single double bond in one of the terminal rings. These pigments can be separated on a $ZnCO_3$-$MgCO_3$ column and the separation requires several hours (*80*) but they are completely resolved in this chromatographic system. However lutein is barely resolved from mutatoxanthin (peak 13) even though mutatoxanthin contains an extra 5,8 epoxide group. Phytofluene and α-cryptoxanthin (peak 19 and 18, respectively) were not well resolved chromatographically, but could easily be separately quantified using different monitoring wavelengths as their respective absorbance maxima differ by approximately 100 nm.

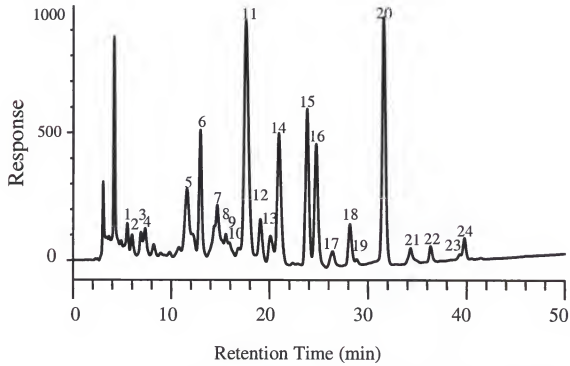

Figure 3-3. Chromatogram of saponified carotenoid extract from orange juice separated using a YMC C30 reverse phase carotenoid column and a water, MeOH, MTBE ternary solvent gradient. Spectral characteristics for each numbered peak are summarized in Table 3-1. See HPLC experimental section for additional details.

Carotenoid Identification

Shown in Figure 3-4 is an overlay of peaks 4, 12 and 24. The height of their spectra corresponds to their relative peak heights since the spectra were taken from the apex of each peak. These were chosen to show the range and diversity of these spectra which are not conveyed when just tabulated peak maxima are tabulated. The shape of the absorbance band as well as the location of the absorbance maxima are all highly characteristic of individual carotenoids. This information taken with retention time can

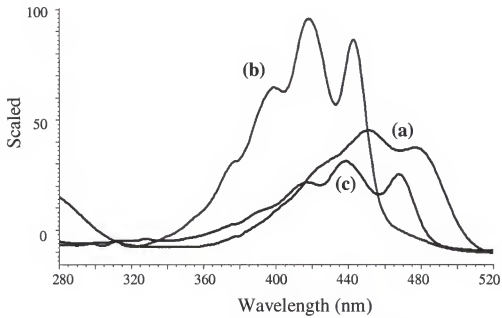

Figure 3-4. Absorbance spectra for β-carotene (a), leutoxanthin (b), and neoxanthin (c), peak 24, 12 and 4 respectively.

be used to identify specific carotenoids, especially if their spectral and chromatographic characteristics have been reported elsewhere. The spectra and relative retention times of α-, β-carotene, α-, β-cryptoxanthin and neoxanthin matched their published values and were used as confirmation of the presence of these peaks in orange juice. It should be pointed out that these five carotenoids have been previously reported in orange juice (*17*).

As shown in Table 3-1 the absorbance maxima observed exactly matched those published in the literature or differed at most by 2 nm as in the case of the central peak for β-cryptoxanthin. Since the wavelength accuracy of most photodiode array detectors is only ± 1 nm, the agreement is excellent. Since the carotenoids of interest have the same elution and spectral characteristics as α-, β-carotene, α-, β-cryptoxanthin and neoxanthin, it is reconfirmed that they are present in orange juice and could potentially serve as norisoprenoid precursors.

Table 3-1. HPLC retention times, spectral characteristics of orange juice carotenoids

Peak no.	Carotenoid	RT [a] (min)	Observed (nm)			Literature (nm)			Ref.[b]
			Peak 1	Peak 2	Peak 3	Peak 1	Peak 2	Peak 3	
1	Valenciaxanthin	5.52	351	369	390	351	369	390	E
2		6.00	371	391	414				
3		6.92	420	435	465				
4	Neoxanthin	7.35	416	438	468	415	439	467	A
5		11.60	410	431	454				
6		12.95	416	438	467				
7	Neochrome	14.68	400	422	448	399.5	421.5	447.5	B
8		15.55	408	429	415				
9		15.90	383	402	425				
10		16.83	411	430	462				
11	Cis-violaxanthin	17.60	415	437	464	414	437	464	C
12	Leutoxanthin	19.07	399	418	443	399.5	419.5	441.5	B
13	Mutatoxanthin	20.08	405	429	451	404	427	452	D
14	Lutein	20.92	420	445	471	424.5	445.5	471.5	B
15	Zeaxanthin	23.80	425	450	476	425	450	478	A
16	Isolutein	24.70	418	441	468	418	439.5	467.5	B
17		26.40	429	445	469				
18	α-cryptoxanthin	28.15	420	445	472	420	444	472	D
19	Phytofluene	28.83	331	348	367	331	348	367	A
20	β-cryptoxanthin	31.57	425	451	477	425	449	476	A
21	β-carotene,5,8: 5',8'-diepoxide	34.30	380	400	424	380	400	425	A
22	α-carotene	36.33	420	446	472	420	445	472	D
23	ζ-carotene	39.28	379	401	425	378	400	425	A
24	β-carotene	39.77	425	451	477	425	450	478	A

[a]RT = retention time, [b]A= Britton (*81*); B= Rouseff et al. (*68*); C= DeRitter and Prucell (*82*); D= Farin et al. (*83*); E= Curl and Bailey (*84*).

Conclusions

Carotenoids in Valencia orange juice were extracted using mixed solvent (hexane:acetone:ethanol, 50:25:25) and subsequently saponified. The saponified carotenoids were separated using a C-30 carotenoid column with a ternary gradient solvent system. Twenty-four carotenoids were separated as distinct peaks and sixteen of these peaks were identified based on their spectral characteristics (Table 3-1), relative elution order compared to literature values and authentic standards. Although they have been reported previously, the presence of α-cryptoxanthin, β–cryptoxanthin, α-carotene, β-carotene and neoxanthin in orange juice was confirmed by comparing retention and spectral properties with standards or literature values. These specific carotenoids were of interest because they possess the direct structural segments needed to serve as precursors potent aroma norisoprenoids.

CHAPTER 4
IDENTIFICATION OF NORISOPRENOIDS IN ORANGE JUICE USING TIME INTENSITY GC-O AND GC-MS

Introduction

Early GC-O studies (*4, 6, 7*) have shown that many aroma active compounds in orange juice exist as low-level volatiles that are difficult to detect using typical FID or MS detectors. Furthermore, these studies also demonstrated that the orange juice volatiles present in highest concentration have little to no aroma activity. Recently, the 25 most intense aroma active compounds in fresh juice, as determined by dilution analysis (*5*), were quantified using isotope dilution analysis (*5*). Beta-ionone is the only orange juice norisoprenoid, which has been fully identified (*4, 5*). Even though it has a moderately intense aroma, it was not one of the 25 odorants recently quantified using isotope-dilution analysis (*5*). Norisoprenoids are volatile C_9-C_{13} fragments with extremely low aroma thresholds which can be formed from the degradation of C_{40} carotenoids. This degradation can be the result of *in vivo* enzymatic reaction, or post harvest thermal degradation in foods containing carotenoids. They are also observed from the release of glycosidically bound norisoprenoids which were originally from carotenoid decompositions as in the case of wine (*58*). Norisoprenoids have been shown to have significant aroma impact in fruits, vegetables and spices such as grapes (*8*), apples (*9*), lychee (*10*) starfruit (*11*), mango (*12*), tomato (*13*), saffron (*14*) cured tobacco (*15*) and black tea (*16*). During the ripening of red raspberries, α-ionone and β-ionone increased to produce the characteristic raspberry aroma (*85*). In heated apple juice,

β-damascenone contributed 32% of the total aroma potency of the juice, and only 1.6% of the total aroma potency of fresh (unheated) apple juice (*9*). Safranal is a potent aroma in saffron formed during drying and storage by hydrolysis from picrocrocin, a monoterpene glycoside (*86*). Beta-cyclocitral, β-ionone and β-damascenone were detected in fresh tomato. Only β-ionone and β-damascenone are the important to tomato aroma because their concentrations (4 and 1 μg/L respectively) are higher than their odor threshold (0.007 and 0.002 μg/L respectively). Beta-damascenone shows a ~ 10-fold increase in concentration in heated tomato juice which was concentrated to tomato paste (*50*). Buttery et al. (*41*) examined both low carotene and high β-carotene tomato lines for norisoprenoids. They found that the high β-carotene line contained the highest concentrations of β-ionone and β-cyclocitral. Both norisoprenoids are known biological or chemical degradation products of β-carotene.

Carotenoids are widely distributed in the plant kingdom and orange juice is particularly rich and a complex source of these compounds (*87*). Lutein, zeaxanthin, β-crytoxanthin, α-carotene and β-carotene have been determined in unsaponified orange juice carotenoids extracted by ethyl acetate (*73*). Thirtynine carotenoid pigments were separated and identified in saponified orange juice carotenoids using HPLC (*68*). Among these, β-carotene, α -carotene, neoxanthin , β-crytoxanthin, lutein, violaxanthin, and canthaxanthine have the structural potential to form potent norisoprenoid fragments (*18-22*). These carotenoids have been confirmed to be present in the Valencia juice used in this study based upon HPLC retention time and spectral characteristics data. (See Chapter 3)

Objectives

Since orange juice has so many carotenoids that could serve as precursors for a wide range of norisoprenoids, the objective of this study was to determine if more than one aroma active norisoprenoid was present in fresh or heat-treated orange juice. If additional norisoprenoids are found, they should be characterized and identified. (See Objective #2)

Materials and Methods

Orange Juice Samples and Processing

Late-season Valencia oranges (from Haines City Citrus Growers Association, Haines City Florida) were juiced using an FMC juice extractor at the Citrus Research and Education Center (CREC), Lake Alfred, Florida. The oranges were juiced using a commercial FMC juice extractor model 291 with standard juice settings. An FMC model 035 juice finisher (FMC Corp., Lakeland, FL) was used with a 0.02 inch screen. The finished juice had a Brix value of 11.7°, an acid content of 0.67% citric acid, a Brix/acid ratio of 17.5 and an oil level of 0.0196%. The freshly squeezed juice was divided into three groups. In Group 1, fresh orange juice was immediately chilled and NaCl (36 g/100 mL of juice) was added to inhibit enzymatic reactions. In Group 2, fresh orange juice was pasteurized using UHT/HTST lab Microthermics tubular pasteurizer Model 25 (Microthermic Corp., Raleigh, NC) at 195°F (90.5°C), held for 12 seconds and filled at 41°F (5°C). The oil level of the pasteurized juice was 0.0168%. In Group 3, fresh orange juice was concentrated to 65° Brix using a thermally accelerated short-time evaporator (TASTE) built by Cook Machinery, Dunedin, Florida. The concentrate was then reconstituted to 11.73° Brix by diluting with deionized water, but without restoring volatiles.

Chemicals

Standard aroma compounds were obtained from the following sources: methional, ethyl 2-methylpropanoate, ethyl 2-methylbutyrate, 1-octanol, 2-acetyl-2-thiazoline, (E,Z)-2,6-nonadienal, (E)-2-nonenal, (E,E)-2,4-decadienal, 1-octen-3-one, ethyl hexanoate, (E,E)-2,4-nonadienal, L-carvone, E-2 octenal, terpinolene, α-terpinyl acetate, α-terpineol, Z-4-decenal, neral, geranial, 4,5-epoxy-E-2-decenal, β-ionone and β-cyclocitral were purchased from Aldrich (Milwaukee, WI). Octanal, limonene, linalool, nonanal, hexanal, decanal, dodecanal, 1,8 cineole, citronella, terpinen-4-ol, β-sinensal, β-myrcene, nootkatone ethyl butyrate, acetaldehyde, geraniol, and nerol were obtained as gifts from SunPure (Lakeland, FL). Alpha-ionone was obtained as a gift from Danisco (Lakeland, FL). The (Z)-2-nonenal was found in purchase of (E)-2-nonenal at the 5-10% level. The (E,Z)-2,4-nonadienal and (E,Z)-2,4-decadienal were found in the purchase of (E,E)-2,4-nonadienal and (E,E)-2,4-decadienal respectively. Their identities were confirmed by mass spectra, retention indices and odor qualities. Beta-damascenone and p-1-Menthen-8-thiol were obtained from Givaudan (Lakeland, FL). The 4-mercapto-4-methyl-2-pentanone and 4-mercapto-4-methyl-2-pentanol were synthesized in our laboratory. The 3-mercaptohexan-1-ol was bought from Interchim (Montlucon, France).

Orange Juice Headspace Extraction

A 10 mL aliquot of orange juice was added to a 40 mL glass vial containing a micro stirring bar and sealed with a screw-top cap that contained a Teflon-coated septa. The bottle and contents were placed in a combination water bath and stirring plate set at 40°C, and gently stirred. After equilibrating for 45 min a SPME fiber (50/30 mm

DVB/Carboxen/PDMS on a 2 cm StableFlex fiber, Supelco, Bellefonte, PA) was inserted into the headspace of the sample bottle and exposed for 45 min. The fiber was then removed from the headspace and inserted into the heated GC injector port at 220°C where the volatiles were thermally desorbed for 5 min.

Gas Chromatography: GC-FID and GC-Olfactometer

Separation was accomplished with a HP-5890 GC (Palo Alto, CA) using either a DB-wax column (30 m x 0.32 mm. i.d. x 0.5 mm, J&W Scientific; Folsom, CA) or Zebron ZB-5 column (30 m x 0.32 mm. i.d. x 0.5 mm, Phenomenex, Torrance, CA). Column oven temperature (for DB-wax) was programmed from 40 to 240°C (or 40 to 265°C for ZB-5) at 7° C/min with a 5 min hold. Helium was used as carrier gas at flow rate of 1.55 mL/min. Injector and detector temperature were 220°C and 290°C, respectively. A narrow-diameter injection port liner (0.75 mm.) was used to improve peak shape and chromatographic efficiency for SPME thermal desorption. The entire separation was conducted in the splitless mode. A GC splitter (Gerstel, Baltimore, MD) split the column effluent between the FID and olfactometer (equipped with a high-volume sniffing port, DATU, Geneva, NY) in a 1:2 ratio, respectively as described by Bazemore et al. (*6*). A time-intensity approach was used to evaluate odor quality and intensity at the sniffing port during the GC run. Assessors rated aroma intensity continuously throughout the chromatographic separation process using a linear potentiometer that supplied a continuous signal to the chromatographic software. Retention times and verbal descriptors were recorded to permit aroma descriptors to be coupled with computerized aroma time-intensity plots. Two olfactometry panelists were trained in GC-sniffing with standard solution of 11 compounds typically found in orange juice (ethyl butanoate, cis-3-hexenol, tran-2-hexenal, α-pinene, myrcene, linalool,

β-citronellol, carvone, terpin-4-ol, geranial, and neral). The panelists sniffed the effluent of aroma standard from GC-O with optimum positioning and breathing technique. The intensity of each standard was recorded on a sliding scale (varying from none to strong intensity) and panelists were provided verbal descriptors of aroma quality. For additional experience, the extract of aroma volatiles from commercial orange juice was provided to panelists under identical conditions. Panelists were accepted on they demonstrated an ability to replicate aroma peak times for at least 80 % of the components in the test mixture.

Two trained panalists evaluated the volatiles of orange juice (extracted by SPME) in duplicate, thus producing four individual time-intensity aromagrams. Average intensity from the four runs was calculated for each odorant. If no peak was detected in a run, its value was treated as missing, not zero. An indication of aroma activity with similar aroma descriptors, at the same retention was required from at least half the panel results before a peak could be considered aroma-active. Averaged time-intensity aromagrams were constructed by plotting average intensity versus retention time. Chromatograms and aromagrams were recorded and integrated using Chromperfect version 5.0, Justice Laboratory Software (Palo Alto, CA). Identification of the aroma-active components was based on the combination of sensory descriptors, standardized retention indices, and identification confirmed by comparison with standards and GC-MS spectra.

Gas Chromatography-Mass Spectrometry

Orange juice headspace volatiles were extracted by SPME and introduced to the GC-MS. Volatiles were separated and analyzed using a Finnigan GCQ ion trap mass

spectrometer (Finnigan, Palo Alto, CA) equipped with a DB5, 60M x 0.25 mm I.D., capillary column (J&W Scientific, Folsom, CA). The injector temperature and transfer line temperature were 200 and 250 °C, respectively. Helium was used as the carrier gas at 1 ml/min. The oven temperature program consisted of a single thermal gradient from 40 to 275 °C at 7°C/min. The MS was set to scan from mass 40 to 300 at 2.0 scans/s in the positive ion electron impact mode. The ionization energy was set at 70 eV.

Aroma Peak Identification

Initial identification was based on the combination of matches with standardized alkane retention index values (Kovat's Index) using two dissimilar column materials (e.g., DB-wax and ZB-5) and aroma characteristics. If the aroma component was sufficiently concentrated, fragmentation patterns were compared with library spectra (NIST 2002 and Wiley (6th Edition) databases using the spectral fit criterion. Only those compounds with spectral fit values equal to or greater than 800 were considered as possible identification candidates. Whenever standards could be obtained, they were used as a confirmation of identification, by comparing the resulting fragmentation pattern, retention index value and aroma descriptor (*88*).

Results and Discussion

Extraction and Concentration of Juice Norisoprenoids

Solid phase microextraction (SPME) was used to extract and concentrate orange juice volatiles because it is a rapid, solventless headspace sampling technique (*6*). When solvent extraction was used, early eluting peaks were obscured by the large quantities of solvent. Early eluting aroma peaks such as acetaldehyde have been shown to be important in orange juice flavor (*89*) but could not be examined using GC-O in solvent-extracted samples. Although solvent extraction would not have presented a problem in

determining norisoprenoids, as they elute fairly late, one of the secondary objectives in the overall study was to determine the relative contribution of norisoprenoids to the total aroma of orange juice. Solvent extracted juice samples would have been unsatisfactory for this purpose for the above stated reason.

The application of headspace SPME to flavor volatile compounds has been employed in the study of flavor volatiles in tomato and strawberry fruits using PDMS, PDMS/DVB, and Carbowax/DVB coated fiber (*67*), in orange juice using a PDMS coated fiber (*64*), a Carboxen-PDMS fiber (*6*), a DVB/Carboxen/PDMS fiber (*65*), PDMS and polyacrylate fiber (*66*). The partition coefficients of the polymeric coatings for the analyses differed markedly. For example, terpenes such as α-pinene, β-myrcene, γ-terpinenes, and limonene are all nonpolar, and were extracted to a higher degree into the nonpolar PDMS coating (*66*). Corresponding PDMS extracted the least amount of the more highly polar volatiles, PDMS/DVB and Carbowax/DVB had partition coefficients higher than that of PDMS for the most polar molecules (*67*). The Carboxen-PDMS fiber coating was more selective for terpenes than early eluting alcohols and aldehydes (*6*). Polyacrylate was more effective in extracting highly polar compounds such as methanol and ethanol (*66*). Due to the wide range of volatile compounds from orange juice and for the increased fiber capacity, the headspace volatiles in this study were extracted and concentrated using the 50/30 mm DVB/Carboxen/PDMS coating on a 2 cm StableFlex fiber.

In examining adsorption curves for β-cyclocitral, β-damascenone and α- and β- ionone on the chosen fiber (see Fig. 5-1) it was concluded that 45 min. represents a rough compromise for all four analytes between minimal exposure time and maximum

peak area. For example β-damascenone and β-ionone reaching more than 80% of their final equilibrium value within 45 min. It is a rare SPME analysis that employs true equilibrium exposure time. If exposure time can be carefully controlled, then exposure times of as little as 5 min. can be employed. These very short exposure times are usually limited to analytes in relatively high concentration and even then the reproducibility is not that good. In this study, very short exposure times were not an option as the analytes of interest were present in very low concentration.

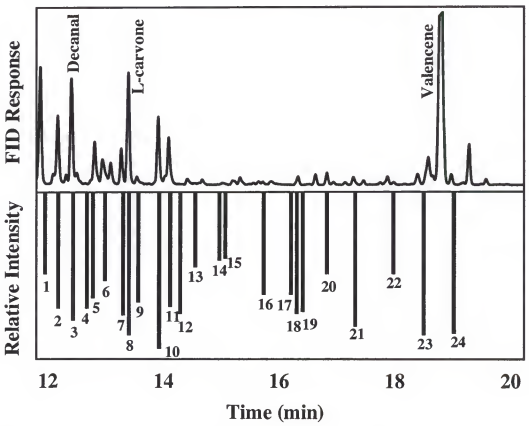

Figure 4-1. GC-FID (top) and average time-intensity of four GC-O runs by two panelists (inverted, bottom) of fresh orange juice on ZB-5 column. Peaks 5, 19, 21 and 23 correspond to norisoprenoids, all numbers refers to compounds in Table 4-1

GC-Olfactometry

In this study, a total of 59 aroma active components were detected in SPME headspace samples from fresh orange juice (orange juice group 1) Since the primary goal of this study was to determine if additional aroma active norisoprenoids were present in orange juice, GC-O was employed primarily in the region where β-ionone and other norisoprenoid standards eluted. Using standards of β–cyclocitral, α-ionone, β-ionone and β-damascenone, the retention time region was established between 12 and 20 min, and the resulting aromagram and concurrent chromatogram is shown in Fig. 4-1.

As noted in Fig. 4-1, four aroma peaks corresponding to peaks 5, 19, 21 and 23 were observed at the identical retention times as β-cyclocitral, β-damescenone, α-ionone and β-ionone respectively. It is also apparent from the relative intensities shown in Table 3-1, that these potential norisoprenoid peaks were among the more intense aromas. Beta-ionone was the most intense and β-cyclocitral was the weakest aroma compound of all the four potential norisoprenoids observed. When the samples were rerun on a DB-wax column the four aroma peaks also were found at retention index values that corresponded with the four potential norisoprenoids. Furthermore, the aroma quality of each juice norisoprenoid corresponded exactly with the aroma description of standards. Since these compounds have the same retention behavior on two very dissimilar chromatographic columns and also have the same aroma quality as standards, they are probably β-cyclocitral, β-damescenone, α-ionone and β-ionone respectively. This represents the first time that β-cyclocitral, and α-ionone have been reported in orange juice. Beta-damescenone had recently been reported in heated orange juice but its identity was not confirmed by supporting instrumental methods (*65*).

Table 4-1. Identification, retention characteristics and aroma descriptions of aroma active compounds in fresh orange juice

No.	Compound	Aroma descriptor	Linear retention		Relative[c] intensity
			ZB-5	DB-wax	
1	Terpinen-4-ol[b]	Metallic, musty	1175	1619	5
2	Z-4-decenal[a]	Green, metallic, soapy	1188	1542	7
3	Decanal[b]	Green, soapy	1198	1508	7
4	(E,E)-2,4-nonadienal[a]	Fatty, green	1209	1702	7
5	β-cyclocitral[b]	Mild floral, sweet, hay-like	1214	1632	6
6	Nerol[a]	Lemongrass	1222	1798	5
7	Neral[b]	Lemongrass	1236	1692	7
8	L-carvone[b]	Minty	1242	1747	8
9	Unknown	Metallic/ woody	1247		6
10	Geraniol[a]	Citrus, geranium	1265	1853	9
11	Unknown	Soapy, almond	1274		7
12	1-p-menthene-8-thiol[a]	Grapefruit	1281	1619	7
13	(E,Z)-2,4-decadienal[a]	Metallic, geranium	1293	1759	4
14	Geranial[a]	Green, minty	1310	1742	4
15	(E,E)-2,4-decadienal[a]	Fatty, green	1314	1819	4
16	α-terpinyl-acetate[a]	Sweet	1349	1663	6
17	4,5-epoxy-E-2-decenal[a]	Metallic, fatty	1375	2010	6
18	Unknown	Sweet nutty	1380		7
19	β-damascenone[b]	Tobacco, apple, floral	1383	1829	7
20	Dodecanal[a]	Soapy	1403	1722	5
21	α-ionone[b]	Floral	1426	1863	8
22	Unknown[a]	Fermented, rancid butter	1459		5
23	β-ionone[b]	Floral, raspberry	1484	1951	8
24	Unknown	Nutty	1510		8

[a] Identified by linear retention index on ZB-5 and/or DB-wax, aroma description as compared with standard

[b] Identified by linear retention index on ZB-5 and/or DB-wax, aroma description as compared with standard, and MS

[c] Averages of normalized intensities (10) evaluated by two trained panelists in four replications

Mass Spectrometry Norisoprenoid Identifications

Headspace volatiles from fresh orange juice were analyzed using capillary GC with an ion trap mass spectrometer. To achieve greater selectivity for the norisoprenoids of interest, selected ion chromatograms were reconstructed in the retention region where norisoprenoid standards were found to elute. The selectivity achieved is demonstrated in Fig. 4-2. Specific m/z values were evaluated to provide the best peak height for each norisoprenoid of interest as well as minimizing interference from non-norisoprenoid

components as well as noise. The following ions were monitored for the specific norisoprenoids: β-cyclocitral, m/z = 137 and 152; β-damascenone, m/z = 175 and 190; α-ionone, m/z = 177 and 192; β-ionone, m/z = 177 and 192.

Although only a single ion has been shown for each norisoprenoid, two or more selective ions were employed to detect the presence of specific norisoprenoids. For example, the selected ion chromatogram using m/z 137 was more intense than that from m/z = 152 but not as specific for β-cyclocitral. The selected ions of m/z = 177 and 192 were extracted for the determination of α-ionone and β -ionone. Selected ion chromatograms at m/z 177 provided excellent signal strength and selectivity for β -ionone. The α-ionone was obviously present at much lower concentrations than β-ionone. The SIC chromatogram at m/z = 192 (Fig. 4-2) provides more selectivity for α-ionone but better signal and noise ratio was obtained at m/z 177.

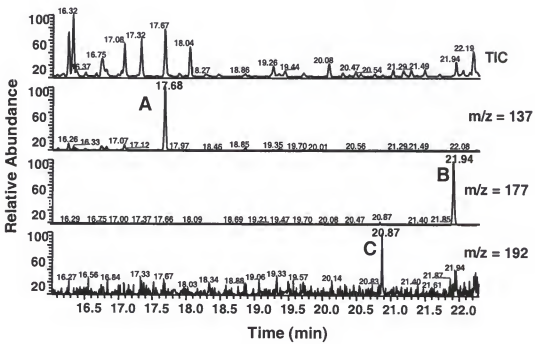

Figure 4-2. Comparison between total ion chromatogram and selected ion chromatograms (SIC) A: β-cyclocitral, B: β–ionone, C: α–ionone.

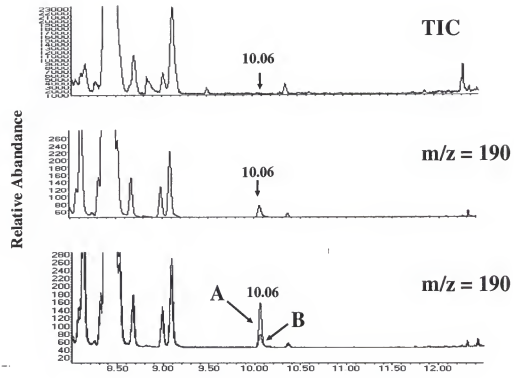

Figure 4-3. Upper, total ion current chromatogram from orange juice headspace, other chromatograms using SIM at m/z 190. Middle chromatogram β-damascenone detected from orange juice, and lower overlay chromatogram of spiked (A) and non-spiked (B) of orange juice with standard β-damascenone.

The β-damascenone, selected ion chromatograms using m/z = 175 and 190 (two masses highly characteristic for β-damascenone) did not provide a clear signal at the expected retention time of β-damascenone using the ion trap MS. Beta-damascenone had been detected by GC-O at the expected retention time with the characteristic aroma but not detected by either FID or SIC ion-trap MS, suggesting that β-damascenone, if present, was there at very low levels. Beta-damascenone has an extremely low odor threshold, which is below the detection limits of most instrumental detectors (0.002 μg/L). However, by employing quadrupole mass spectrometer in the single ion

monitoring mode at least a 10x greater sensitivity (lower detection level) can be achieved because all the ions of a single mass are continuously measured rather than measured for an instant before monitoring other masses. Using selected ion monitoring m/z values 175 and 190, β-damascenone was detected at the expected retention time (see Fig. 4-3 for the case at m/z 190). The combined GC-O and two SIM peaks at the exact retention time of β-damascenone, confirm its presence in orange juice.

Although selected ion chromatograms strongly suggest the presence of the other norisoprenoids of interest, they do not offer absolute proof. They only indicate that a juice volatile elutes at the identical retention time as the norisoprenoid of interest, and this volatile contains the same mass fragment. The combination of this information with the GC-O information provides three independent pieces of information strongly suggesting the presence of specific norisoprenoids. However, to absolutely confirm the presence of β-cyclocitral, α-ionone, and β-ionone, their spectra from the juice MS data at the retention times of each respective norisoprenoid was obtained and compared with reference spectra in standard libraries or compared with that obtained from authentic standards. The resulting match for the case of β-cyclocitral is shown in Fig. 4-4. It is readily apparent that although the relative ion abundances are not the same (usually a function of instrument to instrument variation) an excellent spectral match has occurred and that the presence of β-cyclocitral in orange juice is confirmed. Comparing the relative abundances of ions m/z 137 vs 152 in the upper spectrum of Fig. 4-4, it can be readily appreciated why examining the selective ion chromatogram at 137 provided a better signal to noise ratio than the chromatogram at 152, the molecular ion for β-cyclocitral.

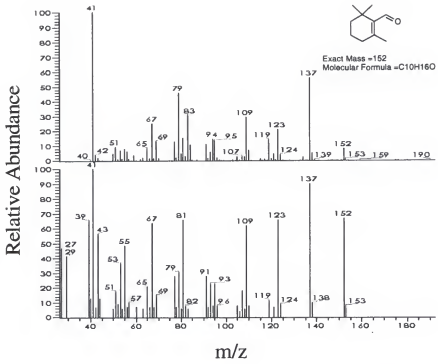

Figure 4-4. Upper spectra from orange juice MS at RT = 17.68 , bottom spectra of β-cyclocitral from database NIST 2002.

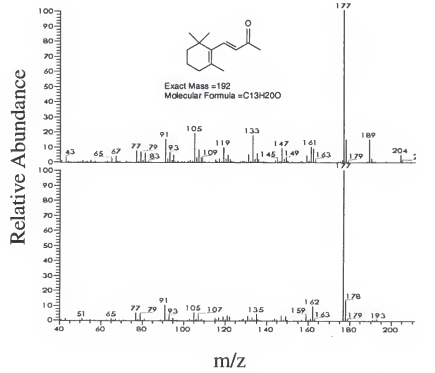

Figure 4-5. Upper spectra from orange juice MS at RT = 21.94, bottom spectra from standard β-ionone using identical ion trap MS at identical retention time.

However, α-ionone has not been previously reported and is shown in Figure 4-6. The spectral match in this case is good considering the very low levels of α-ionone present, but not perfect. Even with careful background subtraction (which was done for all the previous spectra as well), there will be a fair amount of extraneous peaks simply due to random noise. However, the major fragment ions of m/z 192 (M^+), 177, 163, 136, 121, 109, 93, 91, and 77 are all present, more than enough to confirm the presence of α-ionone in orange juice.

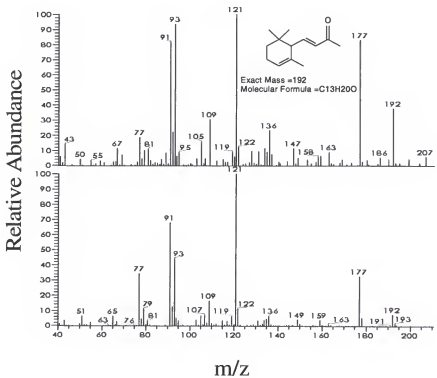

Figure 4-6. Upper spectra from orange juice MS at RT = 20.87, bottom spectra from standard α-ionone using identical ion trap MS at identical retention time.

Conclusion

Four norisoprenoids in fresh orange juice (β-cyclocitral, β-damascenone, α-ionone, and β-ionone) have been conclusively identified through the combined information from

GC-O retention index matches with standards on two dissimilar chromatographic column materials, aroma descriptor matches and GC-MS matches of both retention time and fragmentation spectra. Of these four norisoprenoids, β-ionone had been reported in two previous orange juice GC-O studies (*4, 5*). There is one previous mention of β-damascenone in heated juice, but no MS or independent instrumental confirmation data was presented (*65*). β–cyclocitral and α-ionone were detected in orange juice for the first time in this study and confirming MS data for β-damascenone was presented for the first time.

CHAPTER 5
QUANTIFICATION AND DETERMINATION OF THE RELATIVE IMPACT OF NORISOPRENOIDS IN ORANGE JUICE

Introduction

Chromatographic data is often used to determine the relative concentrations of components in a volatile mixture. Within the linear range of the detector, integrated peak area is proportional to the amount of that component in the sample. The four techniques commonly employed to quantify chromatographic components are normalization; internal standards; external standards; and standard addition methods (*90*). Only a few of these have been employed to determine the amounts of specific volatiles in orange juice volatiles to better understand their contribution to orange flavor (*2, 5*). Buettner and Schieberle (*5*) employed stable isotope dilution assay to quantify 25 volatiles from a solvent extract of hand-squeezed Valencia orange juice. The juice was spiked with a known amount of the labeled internal standard and the juice was extracted with diethyl ether and subsequently analyzed by GC-MS. Standard curves of the labeled and unlabeled reference odorants were used to establish a relationship between peak area and concentration. Odor activity values (OAV, concentrations of the odorants divided by their odor threshold) were determined to estimate their respective odor contributions. The highest OAVs were calculated for (s)-ethyl 2-methylbutanoate, ethyl butanoate, (Z)-3-hexenal, ethyl 2-methylpropanoate, acetaldehyde, and (R)-limonene.

Moshonas and Shaw (*2*) quantified the volatiles from orange juice using dynamic headspace GC with a pressurized purge and trap apparatus. Concentrations for each

volatile were calculated using the standard addition procedure. Regression equations were developed from peak area data from four different concentrations of each compound added to a juice base. Odor activity values were calculated for each component measured (although they were not identified as OAV values). Compounds which exceeded their threshold by the greatest amounts (highest OAV values) and thus most likely to contribute to fresh orange flavor included: limonene, myrcene, α-pinene, decanal, octanal, ethyl butanoate, and linalool. The differences between these two studies which both claim to determine the components most responsible for fresh orange juice flavor are worth noting.

SPME is a rapid, solventless static headspace procedure. It can be used for the quantitative analysis of flavor and fragrance compounds. The standard addition method has been used primarily because the concentration in the headspace (volatility) will be influenced by the sample matrix (*66, 91, 92*). Boa et al. (*92*) reported that reliability problems of headspace SPME quantification is associated with the matrix and could be reduced by employing the standard addition method or employing isotopically labeled internal standards. Headspace SPME with standard additions were used in the present study because SPME can extract and concentrate orange juice headspace volatiles which transfer them directly into the injector of a GC in a simple, straightforward manner. Just as important for this study, the nonvolatile carotenoids will not be extracted. If the nonvolatile carotenoids were present they might degrade when exposed to the heat (200°C) of GC injection port and possibly produce artifact norisoprenoids. The major problem with the standard addition approach is that several injections at each standard addition level are required in order to obtain a single result. Thus, depending on the

number of levels and the number of injections per level, this procedure can be time consuming. However, once a pseudocalibration curve produced, the calculated slope can be used for other samples of similar matrix. Thus, it is not essential that the standard addition be employed for each and every sample, but the slope of the pseudocalibration curve should be checked from time to time. All solutions analyzed must fall within the linear range of the detector response (*90*).

Objectives

The primary goal of this study was to quantify the norisoprenoids in orange juice using static headspace SPME with standard additions and GC-MS. (Objective 4) A secondary goal was to determine the relative aroma contribution of all four norisoprenoids to the total orange juice aroma. (Objective 3)

Materials and Methods

Quantification of Norisoprenoids in Orange Juice

The adsorption (amount vs. exposure time) curves for the SPME fiber (50/30 mm DVB/Carboxen/PDMS) employed in this study was determined by varying exposure time from 5 to 150 minutes. Since native concentrations were so low, orange juice samples were fortified with 8.6, 4, 5.4, and 5.27 ppm β-cyclocitral, β-damascenone, α-ionone, and β-ionone respectively so that adsorption characteristics could be more accurately determined. Ten milliliters of the fortified juice were transferred to 40 ml vial with screw cap coated with Teflon. After 45 min at 40°C, the headspace volatiles were extracted using SPME (as described in Chapter 4)

Headspace SPME and GC-MS were used to quantify norisoprenoids in orange juice using the standard addition method. Each standard (β-cyclocitral, β-damascenone,

α-ionone, and β-ionone) was added separately to the orange juice sample to obtain the final concentration of each norisoprenoid from 0 to 2 ppm. Beta-damascenone was the only exception; its added concentrations ranged from 0 to 0.02 ppm. Sampling was accomplished by adding a 10 mL aliquot of the juice to a 40 ml glass vial containing a micro-stirring bar sealed and a Teflon coated septa. Samples were equilibrated at 40°C for 45 minutes and gently stirred before a SPME fiber was inserted into the headspace of the sample bottle and exposed for another 45 min. The fiber was then removed from the headspace and inserted into the GC-MS. GC conditions were the same as in Chapter 4. Each sample was prepared and injected at lease twice. Quantitative measurements were made using integrated peak areas from selected ion chromatograms. The ions chosen to reconstruct these single ion chromatograms were at m/z 137, 177, 177, and 190 and were fairly unique for β-cyclocitral, β-ionone, α-ionone, and β-damascenone respectively. The latter m/z values corresponded to the respective molecular ion of β-damascenone.

In order to quantify the low levels of β-damascenone, a quadrupole MS (Agilent 5973 Network Mass Selective Detector, Agilent Technologies, CA) was employed using selected ion monitoring (SIM) mode at m/z 190. It was equipped with HP an Innowax 30 m x 0.25 μm x 0.25 μm capillary column (Agilent/J&W HP Innowax, Scientific Instrument Services, Inc., NJ) and autosampler (Gerstel Multi Purpose Sampler MPS2, Gerstel Inc., MD). The oven temperature program consisted of two ramps from 90 to 160°C at 6°C/min and from 160°C to 250°C at 120°C/min (in order to shorter the GC running time after the β-damascenone was eluted). Each sample was analyzed from the response at m/z 190. A graph of SIM 190 peak area versus concentration was prepared

and the amount of β-damascenone in the sample determined from the regression line equation.

Determination of the Relative Impact of Norisoprenoids in Orange Juice

The aroma active compounds from 3 types of orange juice (fresh, pasteurized, and reconstituted from concentrate) were separated and identified using GC-O (chapter 4). Intensities of aroma active compounds of each run were normalized so the highest intensity was given a score of 10. The normalized intensities of all the runs were then averaged, providing a similar aroma activity was detected at least half the time at that retention time. If the compound was not detected in one run its value was treated as missing, not zero. Aroma-active compounds from the entire GC-O trial were categorized into eight groups based on similar aroma description. These eight groups were 1) citrusy/minty; 2) metallic/mushroom/geranium; 3) roasted/cooked/meaty/spice; 4) fatty/soapy/green; 5) sulfury/solventy/medicine; 6) floral; 7) sweet/fruity; and 8) green/grassy. The sums of the total olfactometry intensities for each aroma group was determined and presented in spider web (radar graph) for each of the four juice types. The contribution of norisoprenoids to orange juice was calculated from the total intensity of norisoprenoids to the total intensity of all aroma active volatiles in the juice.

Results and Discussion

Quantification of Norisoprenoids in Orange Juice

The amount of volatile compounds found on the SPME coating depends on exposure time, temperature, sample volume, headspace volume, and sample concentration. In this study only exposure time was varied in order to determine the time needed for equilibrium concentrations for each analyte to be established. All other factors remained constant. Equilibrium time between SPME fiber and headspace of

fortified juices was indicated when little to no increase in peak area was observed with additional exposure time. The equilibrium time for β-cyclocitral, β-damascenone, α-ionone, and β-ionone were 75, 90, 115, and 120 min. respectively (Fig. 5-1). The

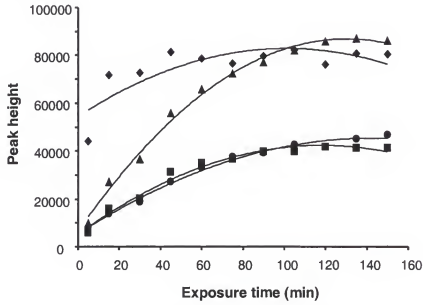

Figure 5-1. Exposure time between SPME fiber and the headspace of orange juice spiked with standards at 40°C, ♦ = β-cyclocitral, ■ = β-damascenone, ▲= α-ionone, ● = β-ionone.

results show that the time needed to reach equilibrium depends on the polarity and the relative molecular mass of each norisoprenoid. Since 75-120 minutes to reach the equilibrium would be too long to wait for practical purposes and may alter the volatile profiles from thermally induced reactions, a shorter exposure time was chosen for routine analyses. It can be seen from the adsorption curves for each compound that 45 min. represents a rough compromise for all four analytes between minimal exposure time and maximum peak area. For example β-damascenone and β-ionone reaching more than 80% of their final equilibrium value within 45 min. It is a rare SPME analysis that employs true equilibrium exposure time. If exposure time can be carefully controlled, then exposure times of as little as 5 min. can be employed. These very short exposure

times are usually limited to analytes in relatively high concentration and even then the reproducibility is not that good. In this study, very short exposure times were not an option as the analytes of interest were present in very low concentrations.

The reproducibility (analytical precision) of a fortified juice using SPME-GC-FID was determined in five replicates at 40°C with 45 min exposure. The relative standard deviations (RSD) obtained were 1.7, 1.7, 0.4, 1.4 % for β-cyclocitral, β-damascenone, α-ionone, and β-ionone respectively (Table 5-1). It should be kept in mind that the orange juice had been fortified with 8.6, 4, 5.4, and 5.27 ppm β-cyclocitral, β-damascenone, α-ionone, and β-ionone respectively. The low RSD indicated that the SPME and GC analytical conditions employed in this study could quantify norisoprenoids in orange

Table 5-1. Reproducibility of SPME exposure time 45 min at 40°C

Replicate	β-cyclocitral	β-damascenone	α-ionone	β-ionone
1	84101	27181	46618	23072
2	84149	26797	46715	22861
3	84129	26534	46813	22589
4	81092	26382	46570	22337
5	82037	25961	46319	22335
Average	83101	26571	46607	22639
STDV[a]	1443	456	186	325
RSD[b]	1.7	1.7	0.4	1.4

[a] Standard deviation, [b] relative standard deviation

juice in a highly reproducible manner. However, it should be pointed out the concentrations used to fortify the sample were considerably higher than would ever be found in an orange juice sample. Typical juice concentrations are 50 to 1000 times lower so that typical RSD's for unfortified juice samples range from 20- 50% which might seem high, but still very acceptable for analyses at the sub µg/L level the complex matrix of orange juice. The volatility of flavor compounds can be changed according to the

sample matrices. Boa et al.(*92*) reported that the combination of SPME with the standard addition method reduce the problem of matrix effects and improved the precision of the procedure.

Norisoprenoid Quantification using Standard Additions

The norisoprenoids β-cyclocitral, β-damascenone, α-ionone, and β-ionone were quantified in orange juice using the standard addition method (Fig. 5-2, 5-3, 5-4, 5-5, and 5-6). The integrate peak areas at specific m/z 137, 177, 177 and 190 for β-cyclocitral,

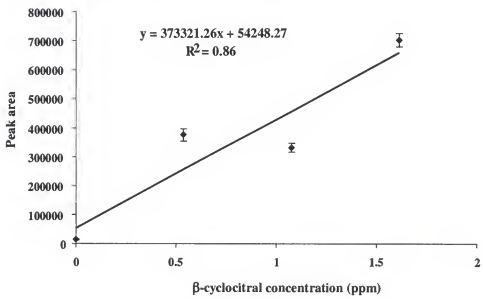

Figure 5-2. Standard addition data for β-cyclocitral peak area vs. added concentration in fresh orange juice. Regression line calculated from peak area at selected mass 137.

β-ionone, α-ionone, and β-damascenone respectively were plotted versus the concentration of the spiked standards. The amount of each norisoprenoid (Table 5-2 and Table 5-3) was calculated from the regression equation where the calculated value was determined at y = 0.

As seen from the plots of Fig. 5-2 through Fig. 5-6, the correlation coefficients for the standard addition data was at least 0.99 in all cases except for β-cyclocitral (Fig. 5-2)

where it was 0.86. One way analysis of variance (ANOVA) show that there are a highly significant differences ($P \leq 0.01$) among orange juice spiked with different concentration of standard α-ionone (Fig. 5-3) and β-ionone (Fig. 5-4). However there were no significant differences within sample. In contrast for β-cyclocitral (Fig. 5-2), there were no significant differences between the two samples spiked with standard β-cyclocitral at concentration 0.54 and 1.1 ppm. This suggests that an error occurred during analysis with at least one data point.

A quadrupole MS provides at least 10x greater sensitivity in the SIM mode than an ion trap MS under the same conditions and was thus used to quantify β-damascenone in fresh and pasteurized juice when the ion trap failed to detect this compound. The calculated slope from the fresh juice data was also employed to determine the concentration of β-damascenone in pasteurized juice as it was thought the matrix effects would be the same for both samples.

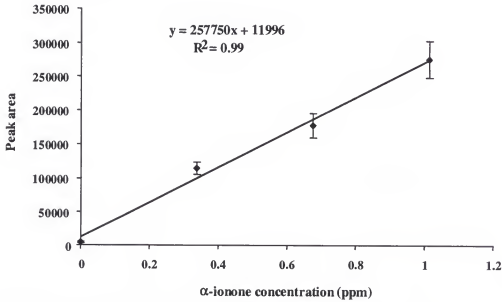

Figure 5-3. Standard addition data for α-ionone peak area vs. added concentration in fresh orange juice. The regression line created by peak area at selected mass 177 vs. α-ionone concentration.

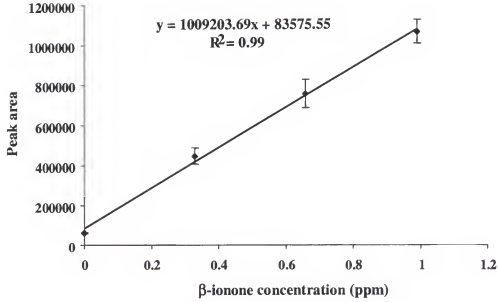

Figure 5-4. Standard addition data for β-ionone peak area vs. added concentration in fresh orange juice. The regression line created by peak area at selected mass 177 vs. β-ionone concentration

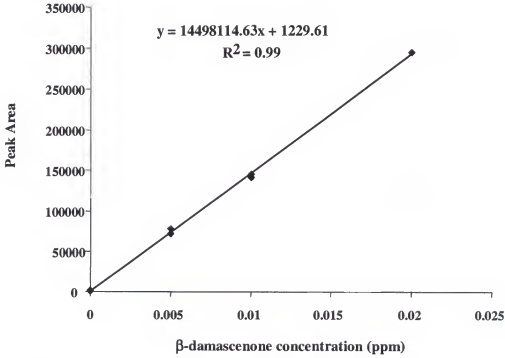

Figure 5-5. Standard addition β-damascenone peak area vs. added concentration in fresh orange juice. GC-quadrupole mass spectrometer in SIM mode at m/z 190.

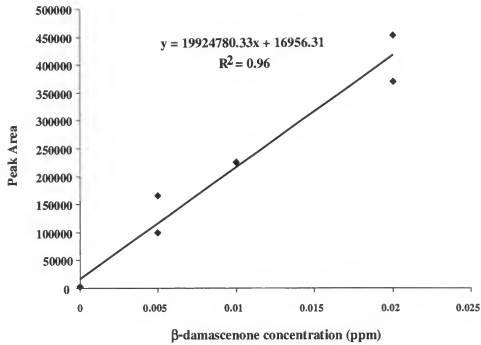

Figure 5-6. Standard addition data of β-damascenone peak area vs. added concentration in reconstituted from concentrate orange juice. GC- quadrupole mass spectrometer in SIM mode at m/z 190.

The concentration of β-damascenone in reconstituted from concentrate was calculated from separate standard addition data (Fig. 5-6) as it was thought that the matrix would be substantially different.

Table 5-2. Concentration of norisoprenoids in fresh orange juice as determinded by standard addition technique

Norisoprenoids	Concentration (μg/L)	Threshold (μg/L in water)[a]	OAV
β-cyclocitral	145	5	25
β-damascenone	0.09	0.002	45
α-ionone	47	0.4	118
β-ionone	83	0.007	11857

[a] Buttery and Teranishi (*50*)

Table 5-3. Concentration of β-damascenone in fresh, pasteurized and reconstituted concentrate

orange juice	concentration (μg/L)	OAV
Fresh	0.09	45
Pasteurized	0.18	90
Reconstituted	0.85	425

The calculated concentrations of β-cyclocitral, β-damascenone, α-ionone and β-ionone in fresh orange juice were 145, 0.09, 47, and 83 μg/L respectively (Table 5-2). The aroma active compounds in orange juice have been studied by GC-O methods (*4-7*). Only β-ionone was reported (*4, 5*) but has not been reported the concentration of this volatile. The concentration of β-damascenone in 3 types of orange juice: fresh, pasteurized, reconstituted from concentrate were 0.09, 0.18, and 0.85 μg/L respectively (Table 5-3). This data suggest that there is precursors present in juice and generate β-damascenone during thermal processing. These precursors are probably carotenoids like neoxanthin, but could also be glycosided forms of β-damascenone. These precursors can generate aroma volatiles in foods that have undergone thermal processing as reported for tomato paste (*23*) and heated apple juice (*9*). It has been previously reported that citrus juice pulp and cloud (insoluble solids) can retain considerable volatiles (*93, 94*). Therefore β-damascenone may have been trapped in the pulp during thermal concentration and might not be completely removed during evaporation. Its partial loss may also been partially compensated by newly β-damascenone generated from thermally unstable carotenoids during thermal concentration.

Determination of Relative Aroma Impact of Norisoprenoids

The odor activity value (OAV) is a rough way of determining relative aroma contribution of various substances. It is determined by dividing the analytical concentration by the aroma threshold. The OAV of β-cyclocitral, β-damascenone, α-ionone and β-ionone were 25, 45, 118, and 11857 respectively (Table 5-2). The OAV value shows that β-ionone is predicted to have the greatest contribution compared to the other norisoprenoids. Hinterholzer and Schieberle, (*4*) analyzed the volatiles from orange

juice by solvent extraction and determined the aroma contribution by aroma extract dilution analysis (AEDA). The value from AEDA was recorded as flavor dilution (FD) factor (the highest dilution factor of the particular aroma active compounds which can be perceived by human nose). The most odor active compound by this method was ethyl butanoate (FD 1024) but the FD factor of β-ionone was only16. These same authors (*5*) quantified twenty-five odor active compounds by stable isotope dilution assay and estimated their respective odor contributions by OAV values. Unfortunately they did not quantify β-ionone. The OAV of ethyl butanoate (the compound with the highest dilution value from AEDA (*4*)) was 1192 (concentration 1192 μg/L, odor threshold 1 μg/L). If one compares this OAV value with the OAV of β-ionone in present study (11857), β-ionone could be the most aroma active compound in orange juice. The apparent conflict in the two sets of data suggests that β-ionone may not have been well extracted in the AEDA study.

Table 5-4. Aroma active compounds in orange juice grouped by citrusy/minty

Compounds[a]	Description	LRI		Relative intensity
		ZB-5	DB-wax	
Unknown	Orange peel	963		8[c], 8[d]
1,8 cineole	Minty, camphor	1026	1232	5[c], 6[d]
Nonanal	Orange peel, soapy	1090	1398	6[c], 6[d]
3-mercapto hexan-1-ol	Grapefruit	1121		7[c], 7[d]
Citronellal	Minty,camphor	1160	1489	5[c], 7[d], 5[e]
Nerol	Lemomgrass	1222	1798	5[c], 5[d]
Neral[b]	Lemomgrass	1236	1692	7[c], 7[d]
L-carvone[b]	Minty	1242	1747	8[c], 8[d]
Geraniol	Citrusy,geranium	1265	1853	9[c], 7[d], 4[e]
1-p-menthene-8-thiol	Grapefruit	1281	1619	7[c], 7[d], 5[e]
Geranial	Green,minty	1310	1742	4[c],4[d], 4[e]
Nootkatone[b]	Sweet,sour, grapefruit	1824		7[c], 5[d]

Table 5-5. Aroma active compounds in orange juice grouped by metallic/mushroom/geranium

Compounds[a]	Description	LRI		Relative intensity
		ZB-5	DB-wax	
1-octene-3-one	Metallic, mushroom	974	1308	6[c], 7[d], 5[e]
β-myrcene	Geranium,plastic	979	1163	7[c], 7[d], 8[e]
Octanal[b]	Metallic, orange peel	998	1299	8[c], 8[d], 8[e]
E-2-octenal	Metallic, fatty, green	1052	1449	4[d]
Terpinolene[b]	Metallic, citrusy	1070	1296	6[c],5[d]
Unknown	Green, metallic	1100		6[d]
Unknown	Metallic, pungent	1128		5[d]
Z-2-nonenal	Green, metallic	1141	1515	4[c], 6[d], 5[e]
Terpinen-4-ol[b]	Metallic, musty	1175	1619	5[c], 6[d]
Unknown	Metallic, woody	1247		6[c], 6[d]
(E,Z)-2,4-decadienal	Metallic, geranium	1293	1759	4[c], 7[d]
4,5-epoxy-E-2-decenal	Metallic, fatty	1375	2010	6[c],6[d], 4[e]
Unknown	Aquarium, metallic	1589		5[c], 6[d]
β-sinensal	Aquarium	1698	2244	8[c], 8[d]

Table 5-6. Aroma active compounds in orange juice grouped by roasted/cooked/meaty/spice

Compounds[a]	Description	LRI		Relative intensity
		ZB-5	DB-wax	
Methional	Cooked potato	904	1464	8[c], 8[d], 7[e]
2-acetyl-2-thiazoline	Cooked jusmine rice	1104	1766	6[c], 6[d], 7[e]
Unknown	Spice	1317		6[d]
Unknown	Sweet, nutty	1380		7[c], 8[d]
Unknown	Fermented, rancid	1459		5[c], 7[d]
Unknown	Green, overipe orange	1461		4[d], 4[e]
Unknown	Nutty	1510		8[c], 9[d]
Unknown	spice	1718		7[c], 7[d]

Table 5-7. Aroma active compounds in orange juice grouped by fatty/soapy/green

Compounds[a]	Description	LRI		Relative intensity
		ZB-5	DB-wax	
Hexanal[b]	Green, fatty	794	1083	7[c], 6[d], 7[e]
1-octanol	Green, soapy	1065	1565	8[c], 7[d], 5[e]
E-2-nonenal	Soapy	1153	1542	8[c], 10[d],6[e]
(Z)-4-decenal	Green, metallic, soapy	1188	1542	7[c], 7[d]
Decanal[b]	Green, soapy	1198	1508	7[c], 8[d], 5[e]
(E,E)-2,4-nonadienal	Fatty, green	1209	1702	7[c], 7[d]
Unknown	Soapy, almond	1274		7[c], 7[d]
(E,E)-2,4-decadienal	Fatty, green	1314	1819	4[c], 6[d]
Dodecanal	Soapy	1403	1722	5[c], 6[d]

Table 5-8. Aroma active compounds in orange juice grouped by sulfury/solventy/medicine

Compounds[a]	Description	LRI		Relative intensity
		ZB-5	DB-wax	
Acetaldehyde	Fresh alcohol	445	732	6[c], 6[d]
Carbon disulfide	Sulfur, fermented cabbage	678		6[c], 7[d], 6[e]
Dimethyl sulfide	Solventy, plastic	691		6[c], 6[d], 4[e]
Dimethyl disulfide	Plastic	772	1074	4[c], 4[d], 7[e]
Unknown	Fermented, sulfur	818		5[c], 6[d]
2-methyl-3-furanthiol	Meaty, vitamin, medicine	865	1305	7[c], 7[d], 6[e]
4-mercapto-4-methyl-2-pentanone	Sulfury, grapefruit	944	1389	7[c], 5[d]
Dimethyl trisulfide	Sulfur, sweaty	968	1392	3[c], 5[d],8[e]
4-mercapto-4-methyl-2-pentanol	Sweaty,grapefruit,guava	1039		7[c], 7[d], 7[e]
Unknown	Solventy	1167		5[d]
Dimethyl tetrasulfide	Sulfury, musty	1225		6[e]

Table 5-9. Aroma active compounds in orange juice grouped by floral

Compounds[a]	Description	LRI		Relative intensity
		ZB-5	DB-wax	
Linalool[b]	Floral	1094	1551	8[c], 8[d], 5[e]
β-cyclocitral[b]	Mild floral, hay-like	1214	1632	6[c], 4[d]
Unknown	Tobacco,sweet, floral	1255		6[e]
β-damascenone[b]	Tabacco, apple, floral	1383	1829	7[c], 8[d], 8[e]
α-ionone[b]	Floral	1426	1863	8[c], 8[d]
β-ionone[b]	Floral, raspberry	1484	1951	8[c], 9[d], 7[e]

Table 5-10. Aroma active compounds in orange juice grouped by sweet/fruity

Compounds[a]	Description	LRI		Relative intensity
		ZB-5	DB-wax	
Ethyl-2-methylpropanoate	Sweet, fruity	758	966	6[c], 6[d]
Ethyl butyrate[b]	Sweet, fruity	795	1034	4[c], 6[d], 8[e]
Ethyl-2-methylbutyrate	Sweet, fruity	846	1051	5[c], 6[d]
Ethyl hexanoate[b]	Sweet	994	1242	6[c], 7[d]
α-terpinyl acetate	Sweet	1349	1663	6[c],6[d]

Table 5-11. Aroma active compounds in orange juice grouped by green/grassy

Compounds[a]	Description	LRI		Relative intensity
		ZB-5	DB-wax	
3-(Z)-hexen-1-ol / (E)-2-hexenal[b]	Green, grass	854	1226/1391	6[c], 7[d]
(E,Z)-2,6-nonadienal	Green	1148	1593	7[c], 8[d], 7[e]

[a] Identified by linear retention index on ZB-5 and/or DB-wax, aroma description as compared with standard
[b] Identified by linear retention index on ZB-5 and/or DB-wax, aroma description as compared with standard, and MS
[c] fresh orange juice
[d] pasteurized orange juice
[e] reconstituted from concentrate

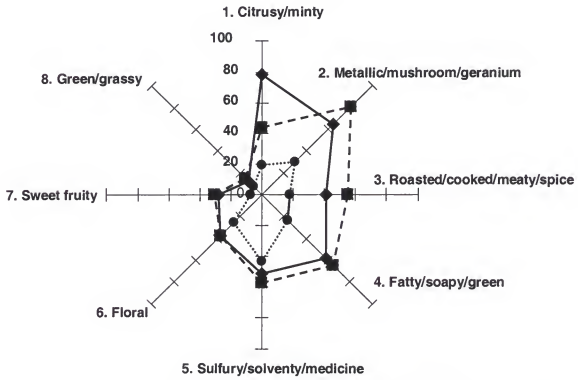

Figure 5-7. Aroma group profiles of fresh (◆), pasteurized (■), and reconstituted from concentrate (●) orange juice.

Norisoprenoid Contribution to Total Floral Aroma

All four of the identified norisoprenoids in orange juice have a general floral aroma quality (Table 5-9). When all the relative intensities of these four norisoprenoids were combined (in each type of orange juice) and compared with the total aroma

intensity, their contribution to total juice aroma were 7.8, 7.6, and 8.7 % in fresh, pasteurized, and reconstituted from concentrate respectively. Fig. 5-7 illustrates aroma group profiles from the total intensity in similar aroma quality groups from GC-O aromagrams, comparing among fresh, pasteurized, and reconstituted from concentrate. Comparing fresh and pasteurized orange juice, the major aroma category differences were in the metallic/mushroom/geranium and in roasted/cooked/meaty/spice categories which were higher in pasteurized juice than fresh juice. This difference come from three addition aroma active compounds (E-2-octenal, and two unknown at LRI 1100 and LRI 1128) in metallic/mushroom/geranium category and two additional unknown (LRI 1317 and LRI 1461) in roasted/cooked/meaty /spice category in pasteurized juice (Table 5-5 and Table 5-6). In fresh juice citrusy/minty category was higher than pasteurized juice. The differences were the higher intensity of compounds in citrusy/minty category in fresh juice than in pasteurized juice. The contribution of floral quality was the same in fresh and pasteurized orange juice. From the aroma group profile of reconstituted from concentrate there were many volatile compounds lost due to the thermal evaporation process.

Fig. 5-8 shows the contribution of aroma active compounds in just the floral category. This group includes linalool, β-cyclocitral, β-damascenone, α-ionone, β-ionone and one unknown (LRI 1255) generated after thermal processing. The norisoprenoids in orange juice contribute the majority of floral aroma in floral category, specifically 78, 78, and 59% in fresh, pasteurized and reconstituted from concentrate respectively.

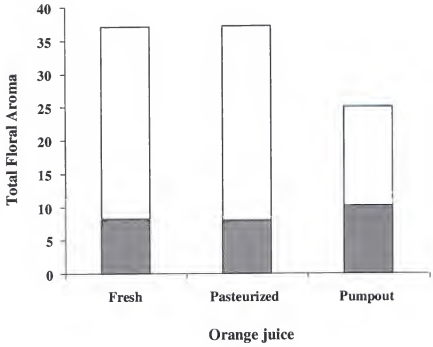

Figure 5-8. Upper bar norisoprenoids contribute mainly to the total floral category, fresh = 78%, pasteurized = 78%, and reconstituted = 59%, lower bar represent non-norisoprenoids including linalool and unknown (LRI = 1255) generated during thermal processing.

Table 5-12. Norisoprenoids in orange juice and peel oil

Norisoprenoids	Fresh	Pasteurized	Reconstituted concentrate	Hand squeezed	Peel oil
β-cyclocitral	X	X		X	X
β-damascenone	X	X	X	X	X
α-ionone	X	X		X	
β-ionone	X	X	X	X	

X = indicates presence of norisoprenoids in the various samples.

Four norisoprenoids, β-cyclocitral, β-damascenone, α-ionone, and β-ionone were detected in both fresh and pasteurized juice (Table 5-12). Only two norisoprenoids β-damascenone and β-ionone were detected in reconstituted from concentrate, indicating that these two compounds could be generated from precursors during thermal evaporation and/or they were retained by the pulp during the evaporation process.

Conclusion

Concentrations of four orange juice norisoprenoids were determined using SPME with the standard addition method. The concentrations of β-cyclocitral, β-damascenone, α-ionone, and β-ionone in fresh orange juice were 145, 0.09, 47, and 83 μg/L respectively. The OAV (determined by dividing the analytical concentration by the aroma threshold) of β-cyclocitral, β-damascenone, α-ionone, and β-ionone were 25, 45, 118, and 11857 respectively. The OAV values suggest that β-ionone provides the greatest aroma contribution compared to the other norisoprenoids. The concentration of β-damascenone increaded with thermal processing, indicating that there are precursors in juice which generate β-damascenone during elevated temperatures. Combined, the four norisoprenoids contribute 8-10% of the total aroma impact. The norisoprenoids have a general floral character and contribute the majority (60-80%) of the floral character to orange juice.

CHAPTER 6
THERMAL DEGRADATION OF BETA-CAROTENE IN MODEL SOLUTION

Introduction

Carotenoids are unstable in both the presence of heat and/or light. The thermal degradation of carotenoids produces a range of volatile products and norisoprenoids are the most potent aroma compounds of all the volatiles produced. The formation of specific norisoprenoids from the thermal degradation of carotenoids during heat treatment of food products have been reported. Beta-ionone, α-ionone, and β-damascenone have been reported in tomato paste (*23*) and black tea (*95*). These norisoprenoids also were detected from the thermal degradation of carotenoids in model systems such as β-carotene in water at 97°C, 3 hrs (*31*), 1% solution of β-carotene heated at 188°F for 72 hrs. (*96*), and thermal degradation of crystallize β-carotene at 240°F in a vaccum (*97*). However the norisoprenoids formed in those model systems were formed at high temperature. At best these studies could be considered accelerated storage studies. In order to have a model more representative of the conditions that a "real world" juice might be exposed to, a 35°C storage study was carried out. The model solution was buffered to pH 3.8 (orange juice pH) using citric acid and tripotassium citrate. Sugars and amino acids were not added to reduce the possibility that they could be possible norisoprenoid sources. The concept that carotenoids could act as a source of norisoprenoids is relatively new (*15, 39, 69, 70*). These studies have indicated that specific carotenoids need be present in order to produce specific norisoprenoids.

Therefore juice carotenoids may be the source of some aromas due to thermal degradation during processing and subsequence storage.

Twenty-four carotenoids from orange juice were isolated in present study (i.e., neoxanthin, β-carotene. α-carotene, and β-cryptoxanthin, see chapter 3). One of the carotenoids isolated in orange juice, β-carotene, was studied in model aqueous solution to determine which aroma active compounds could be produced via thermal degradation and thus unequivocally demonstrate that a carotenoid found in orange juice can act as a precursor of norisoprenoids. Beta-carotene was chosen because it was commercially available, relatively inexpensive and should be the most unstable as it cannot be esterified to improve thermal stability.

Objective

Determine if aroma active norisoprenoids are generated from β-carotene via thermal degradation using model solutions adjusted to orange juice pH (3.8) and stored at 35°C. (Objective 5)

Materials and Methods

Crystallization

Beta-carotene (99% purity, purchase from Acros) was recrystallized before using to remove aroma active impurities. The method of recrystallization followed that of Schiedt and Liaaen-Jensen (*98*) with minor modification. Beta-carotene was dissolved in the smallest possible volume of petroleum ether (Fisher Scientific, NJ), filtered through glass wool in a funnel, and ethanol (Fisher Scientific, NJ) was added drop-wise until turbidity was observed. The mixture was left at room temperature for about an hour and the temperature was then lowered gradually to 6°C (refrigerator) and finally to –20°C (deep-freeze) over night or until the crystals formed. The crystals were collected on a fine

sintered-glass, washed on the filter with cold ethanol and dried with the flow of nitrogen gas. The headspace volatiles of recrystallized β-carotene were checked by SPME before using. It was ready to use when no aroma active volatiles were detected.

Model Solutions

Acetone (Fisher Scientific, NJ) was chosen to dissolve the recrystallized β-carotene because it was a polar solvent and would facilitate the transfer of β-carotene into the model aqueous solution. One milligram of recrystallized β-carotene was dissolved in acetone and diluted to citrate buffer pH 3.8 (citric acid 1.2 g., tripotassium citrate 0.6 g. adjust pH to 3.8 by 1 N. NaOH, (Fisher Scientific, NJ)). Ten milliliters of the solution were added into 40 ml vial with Teflon coated screw cap and wrapped with aluminum foil kept in 35°C for up to 1 month.

Analytical Methods

The headspace volatiles of the model solution were extracted by Solid Phase Microextraction (SPME,50/30μm DVB/Carboxen/PDMS, Supelco). The solution was equilibrated at 40°C with gentle agitation (by stirring bar) for 45 min and then inserted the SPME fiber to the headspace of the model solution in order to extract and concentrate the headspace volatile by the fiber for another 45 min. The fiber was injected to GC (A HP-5890 GC (Palo Alto, CA) with either a DB-Wax or ZB-5 column whose effluent was split between an olfactometer or flame ionization detector (FID). Column oven temperature was programmed from 40 to 240°C at 7 °C/min with a 5 min hold. The aroma active compounds detected by GC-O were identified from their aroma quality and retention index by comparison with standards and confirmed by GC-MS (as describer in chapter 4).

Results and discussion

Before beginning storage study, the high purity β-carotene (99% purity) was evaluated for aroma active impurities using GC-O of the material in the same manner as the storage study. This demonstrated the potency of very minor impurities (less than 1%) and the need to recrystallize the standard β-carotene to remove aroma active impurity before beginning the storage study (Fig. 6-1). No effort was made to identify these impurities only to remove them. Freshly recrystallized β-carotene was used in all model solution storage studies. Before storage, a day 0 (control) was examined using GC-O to make certain no aroma active volatiles were detected (Fig. 6-2).

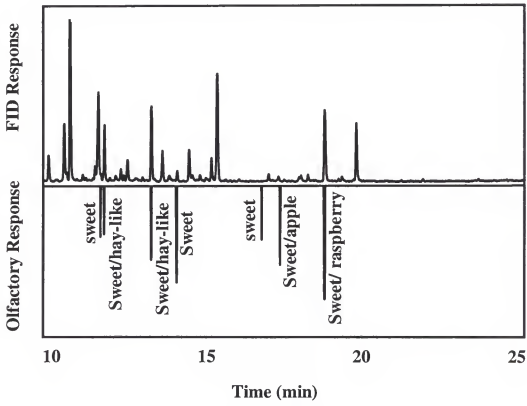

Figure 6-1. The standard β-carotene (99% purity) as received (no purification)

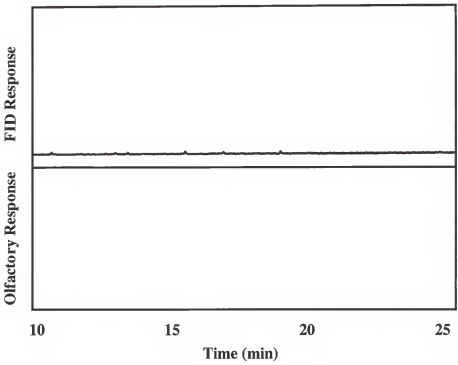

Figure 6-2. Headspace volatiles from β-carotene in model solution pH 3.5 at 0 day

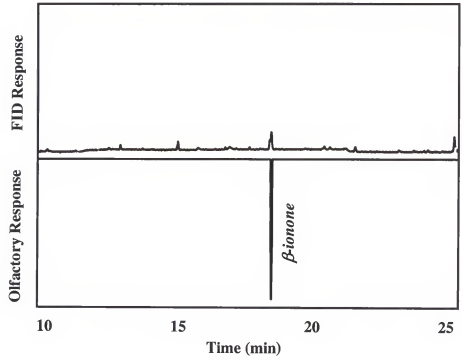

Figure 6-3. Headspace volatiles from β-carotene in model solution pH 3.5 after storage 1 day at 35°C: 1 = β-ionone, a = sweet/raspberry

After one day of storage at 35°C, only a single aroma active compound, β-ionone, was detected (Fig. 6-3). This suggests that β-ionone is either one of the most common thermal decomposition products and/or it has one of the lowest aroma thresholds. Beta-ionone does have one of the lowest aroma thresholds (see Table 5-2), but is probably also a common decomposition fragment and as shown in Fig. 6-3 might represent a certain weakness in the C_9-C_{10} double bond.

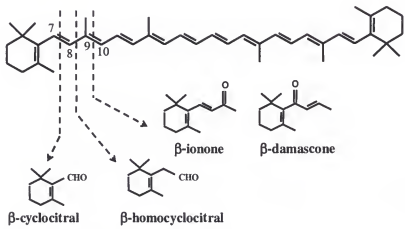

Figure 6-4. Degradation of β-carotene in model solution at difference carbon bonds

After two weeks storage, five distinct aroma active peaks were observed. Four of these appeared to correspond to distinct FID peaks. It is interesting to note that β-ionone is still the highest peak and that all of the predicted decomposition products shown in Fig. 6-4 were observed (e.g., peak 1 was due to β-cyclocitral, peak 2 was due to β-homocyclocitral). It appears that oxidative degradation of β-carotene at double bond C_9-C_{10}, is the most preferable and β-ionone was reported as the major product from β-carotene degradation (*32, 99*). Beta-ionone was reported as an off-flavor of dehydrated carrot stored in oxygen. When dehydrated carrot was stored in the presence of oxygen its

color, due to β-carotene, was destroyed and simultaneously an off-flavor (violet-like) developed (*24*).

The polyene-carotenes are apparently oxidized at the first conjugated diene bonds. Oxidation is more prevalent adjacent to the methyl group and it is possible that inductive effects of the methyl group make the double bond adjacent to it more susceptible to oxidation (*100*). When β-carotene in benzene or tetrachloromethane is allowed to react with molecular oxygen in the absence of light at 30°C, β-ionone can be formed within the first few hours. As the oxidation progressed a number of shorter chain products are formed, including β-cyclocitral (*101*). The aroma active volatile compounds formed indicates that oxidative scission of β-carotene can occur at carbon bond C_7-C_8, C_8-C_9 and C_9-C_{10} of β-carotene to generate C_{10}: β -cyclocitral, C_{11}: β-homocyclocitral , C_{13}: β-damascone and C_{13}: β-ionone respectively (see figure 6-4). The same degradation position and volatile compounds (except β-homocyclocitral) have been reported by photo-oxygenation (*102*) autoxidation with molecular oxygen at 30°C in the dark conditions (*101*) and oxidation in water at 97°C (*31*). At the scission C_8-C_9 of β-carotene, β-homocyclocitral (C_{11}) was formed in present model condition but at the same scission position, dihydroactinodiolide (C_{11}) was formed and has been reported in the different model conditions (*31, 101, 102*).

GC-O Analysis of β-Carotene Decomposition at 35°C

The GC-O data for the two week storage sample is in Table 6-1. It should be noted that the same retention and aroma descriptors observed earlier for standard and juices were also observed for this storage sample. It is interesting to see just how similar the values in Table 6-1 are with their corresponding components in Table 5-9. In making this

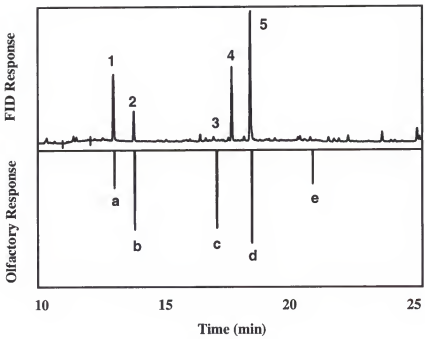

Figure 6-5. Headspace volatiles from β-carotene in model solution pH 3.5, after storage 2 weeks at 35°C : 1 = β-cyclocitral, 2 = β-homocyclocitral, 3 = β-damascone, 4 = unknown, 5 = β-ionone, a = sweet/floral/hay-like, b = sweet/floral/hay-like, c = sweet/apple, d = sweet/raspberry, e = sweet.

Table 6-1. Aroma active compounds from β-carotene thermal degradation in model solution pH 3.8, storage at 35°C for 2 weeks

Compounds	Aroma description	LRI		MS
		ZB-5	DB-wax	
β-cyclocitral	Sweet, floral, hay-like	1228	1632	x
β-homocyclocitral	Sweet, floral, hay-like	1262	1780	x
β-damascone	Sweet, floral	1425	1835	
β-ionone	Sweet, raspberry	1495	1960	x

comparison, it will be noted that neither β-homocyclocitral or β-damascone was found in orange juice. Their retention characteristics and aroma descriptors exactly matched that of authentic standards, providing enough evidence for at least a tentative identification. Positive identification of these compounds was achieved from the MS data. The MS fragmentation patterns for the identified norisoprenoids are shown in the following figures which should offer conclusive proof as to their identity.

MS Identification

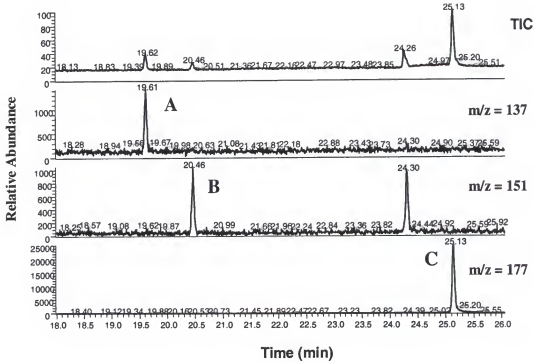

Figure 6-6. Selected ion chromatogram (SIC) of model solution headspace volatiles after storage 2 weeks at 35°C : A = β-cyclocitral, B = β-homocyclocitral, C = β-ionone.

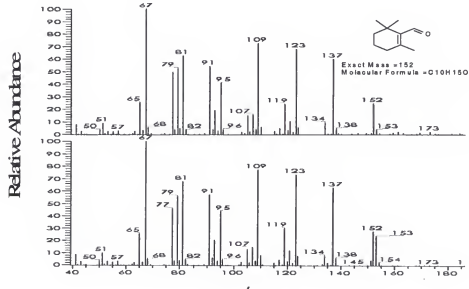

Figure 6-7. Upper spectra from model solution MS at RT 19.61, bottom spectra from standard β-cyclocitral using identical ion trap MS at identical retention time.

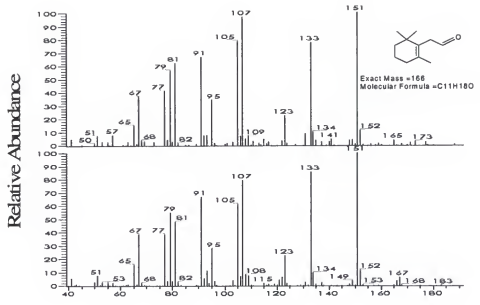

Figure 6-8. Upper spectra from model solution MS at RT 20.46, bottom spectra from standard β-homocyclocitral using identical ion trap MS at identical retention time

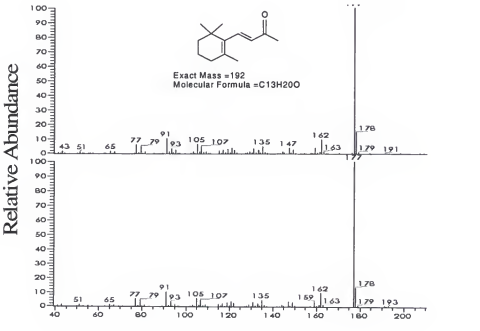

Figure 6-9. Upper spectra from model solution MS at RT 25.13, bottom spectra from standard β-ionone using identical ion trap MS at identical retention time.

Conclusion

The norisoprenoids detected in this study were also detected in other foods that have been thermally processed e.g.,tea (*95*), tomato paste (*50*). Therefore the results of this study indicate that β-carotene could be a precursor of β-cyclocitral and β-ionone in orange juice. Furthermore it could be a precursor of norisoprenoids during thermal processing and subsequence storage at relative high temperature and it is reasonable to assume that any appreciable change in carotenoids content of orange juice will have an effect on flavor.

CHAPTER 7
CONCLUSIONS

Four norisoprenoids, β-cyclocitral, β-damascenone, α-ionone and β-ionone were identified in orange juice using headspace SPME, GC-O, GC-FID and GC-MS. Three of them, β-cyclocitral, β-damascenone, α-ionone were identified and confirmed by GC-MS for the first time. Their concentrations in fresh orange juice were determined using SPME with standard addition technique. Odor activity values (OAV) were calculated using published threshold values. Calculated OAV values suggest that β-ionone provided the greatest contribution to total floral aroma in orange juice compared to the other three norisoprenoids. The concentration of β-damascenone increased almost 10 fold after thermal processing, indicating there are thermally unstable precursors which generate β-damascenone at elevated temperatures. All four of the norisoprenoids in orange juice contribute 8-10% floral aroma to the total aroma quality of the orange juice and are the major contributors (60-80%) in the floral category.

Several carotenoids were identified using HPLC with photodiode array detection. Twenty-four carotenoids were separated as distinct peaks and sixteen of these peaks were identified based on their spectral characteristics, relative elution order compared to literature values and authentic standards. The identified carotenoids include: α-carotene, β-carotene, α-cryptoxanthin, β-cryptoxanthin and neoxanthin, which are known as norisoprenoid precursors. These specific carotenoids were of interest because they

possess the direct structural segments needed to serve as precursors to the newly identified norisoprenoids.

To demonstrate that carotenoids could serve as norisoprenoids precursors, β-carotene was studied in a model system at 35°C storage. GC-O and GC-MS data confirmed the presence of β-cyclocitral and β-ionone in these solutions in as little as two weeks. This was direct proof that β-carotene can degrade to form specific norisoprenoids under conditions an orange juice might encounter.

LIST OF REFERENCES

1 Moshonas, M. G.; Shaw, P. E. Quantitative analysis of orange juice flavor volatiles by direct-injection gas chromatography. *J. Agric. Food Chem.* **1987**, *35*, 161-165.

2 Moshonas, M. G.; Shaw, P. E. Quantitative determination of 46 volatile constituents in fresh, unpasteurized orange juices using dynamic headspace gas chromatography. *J. Agric. Food Chem.* **1994**, *42*, 1525-1528.

3 Tonder, D.; Petersen, M. A.; Poll, L.; Olsen, C. E. Discrimination between freshly made and stored reconstituted orange juice using GC odor profiling and aroma values. *Food Chem.* **1998**, *61*, 223-229.

4 Hinterholzer, A.; Schieberle, P. Identification of the most odor-active volatiles in fresh, hand-extracted juice of Valencia late oranges by odor dilution techniques. *Flavour Frag. J.* **1998**, *13*, 49-55.

5 Buettner, A.; Schieberle, P. Evaluation of aroma differences between hand-squeezed juices from Valencia late and Navel oranges by quantitation of key odorants and flavor reconstitution experiments. *J. Agric. Food Chem.* **2001**, *49*, 2387-2394.

6 Bazemore, R.; Goodner, K.; Rouseff, R. Volatiles from unpasteurized and excessively heated orange juice analyzed with solid phase microextraction and GC-olfactometry. *J. Food Sci.* **1999**, *64*, 800-803.

7 Marin, A. B.; Acree, T. E.; Hotchkiss, J. H.; Nagy, S. Gas chromatography-olfactometry of orange juice to assess the effects of plastic polymers on aroma character. *J. Agric. Food Chem.* **1992**, *40*, 650-654.

8 Kotseridis, Y.; Baumes, R. Identification of impact odorants in bordeaux red grape juice, in the commercial yeast used for its fermentation, and in the produced wine. *J. Agric. Food Chem.* **2000**, *48*, 400-406.

9 Zhou, P. G.; Cox, J. A.; Roberts, D. D.; Acree, T. E. Beta-Damascenone precursors in apples. *Prog. Flavour Precursor Stud. Proc. Int. Conf.* **1993**, 261-273.

10 Ong, P. K. C.; Acree, T. E. Gas chromatography/olfactory analysis of Lychee (*Litchi chinensis Sonn.*). *J. Agric. Food Chem.* **1998**, *46*, 2282-2286.

11 MacLeod, G.; Ames, J. M. Volatile components of starfruit. *Phytochemistry* **1990**, *29*, 165-172.

12 Olle, D.; Baron, A.; Lozano, Y. F.; Sznaper, C.; Baumes, R.; Bayonove, C.; Brillouet, J. M. Microfiltration and reverse osmosis affect recovery of mango puree flavor compounds. *J. Food Sci.* **1997**, *62*, 1116-1119.

13 Ishida, B. K.; Mahoney, N. E.; Ling, L. C. Increased lycopene and flavor volatile production in tomato calyces and fruit cultured in vitro and the effect of 2-(4-Chlorophenylthio)triethylamine. *J. Agric. Food Chem.* **1998**, *46*, 4577-4582.

14 Tarantilis, P. A.; Polissiou, M. G. Isolation and identification of the aroma components from saffron (*Crocus sativus*). *J. Agric. Food Chem.* **1997**, *45*, 459-462.

15 Enzell, C. Biodegradation of carotenoids - an important route to aroma compounds. *Pure Appl. Chem.* **1985**, *57*, 693-700.

16 Sanderson, G. W.; Co, H.; Gonzalez, J. G. Biochemistry of tea fermentation: role of carotenes in black tea aroma formation. *J. Food Sci.* **1971**, *36*, 231-236.

17 Gross, J.; Gabai, M.; Lifshitz, A. Comparative study of the carotenoid pigments in juice of Shamouti, Valencia, and Washington oranges, three varieties of *Citrus sinensis*. *Phytochemistry* **1972**, *11*, 303-308.

18 Isoe, S.; Hyeon, S. B.; Sakan, T. Photo-oxygenation of carotenoids. I. Formation of dihydroactinidiolide and β-ionone from β-carotene. *Tetrahedron Lett.* **1969**, 279-281.

19 Demole, E.; Enggist, P.; Winter, M.; Furrer, A.; Schulte-Elte, K. H.; Egger, B.; Ohloff, G. Megastigma-5,8-dien-4-one, an aroma constituent of the yellow passion fruit and Virginia tobacco. *Helv. Chim. Acta* **1979**, *62*, 67-75.

20 Strauss, C. R.; Wilson, B.; Anderson, R.; Williams, P. J. Development of precursors of C13 nor-isoprenoid flavorants in Riesling grapes. *Am. J. Enol. Vitic.* **1987**, *38*, 23-27.

21 Williams, P. J.; Sefton, M. A.; Francis, I. L. Glycosidic Precursors of Varietal Grape and Wine Flavor. In *Flavor Precursors: Thermal and Enzymatic Conversions*; R. Teranishi; G. R. Takeoka and M. Guntert, Eds.; American Chemical Society: Washington, DC, 1992; pp 74-86.

22 Enzell, C. R. Influence of Curing on the Formation of Tobacco Flavor. In *Flavour '81*; P. Schreier, Ed.; de Gruyter: Berlin, New York, 1981; pp 449-478.

23 Buttery, R. G.; Teranishi, R.; Flath, R. A.; Ling, L. C. Identification of additional tomato paste volatiles. *J. Agric. Food Chem.* **1990**, *38*, 792-795.

24 Ayers, J. E.; Fishwick, M. J.; Land, D. G.; Swain, T. Off-flavor of dehydrated carrot stored in oxygen. *Nature* **1964**, *203*, 81-82.

25 Vernin, G. Terpenoids and norisoprenoids in grape berry aromas and in wines. 1. Origins and formation. *Rivista Italiana EPPOS* **1997**, *8*, 19-31.

26 Gross, J. *Pigments in Fruits*; Academic Press Inc.: London, 1987; 303 pp.

27 Weedon, B. C. L.; Moss, G. P. Structure and nomenclature. In *Carotenoids*; G. Britton; S. Liaaen-Jensen and H. Pfander, Eds.; Birkhauser Verlag: Basel Boston Berlin, 1995; pp 27-70.

28 Mordi, R. C. Mechanism of β-carotene degradation. *Biochem. J.* **1993**, *292*, 310-312.

29 Lutz-Roder, A.; Jezussek, M.; Winterhalter, P. Nickel peroxide induced oxidation of canthaxanthin. *J. Agric. Food Chem.* **1999**, *47*, 1887-1891.

30 Ouyang, J. M.; Daun, H.; Chang, S. S.; Ho, C. T. Formation of carbonyl compounds from β-carotene during palm oil deodorization. *J. Food Sci.* **1980**, *45*, 1214-1217.

31 Kanasawud, P.; Crouzet, J. C. Mechanism of formation of volatile compounds by thermal degradation of carotenoids in aqueous medium. 1. β-Carotene degradation. *J. Agric. Food Chem.* **1990**, *38*, 237-243.

32 Winterhalter, P. Carotenoid-Derived Aroma Compounds: Biogenetic and Biotechnological Aspects. In *Biotechnology for Improved Foods and Flavors*; G. R. Takeoka; R. Teranishi; P. J. Williams and A. Kobayashi, Eds.; American Chemical Society: Washington, DC, 1996; pp 295-308.

33 Weeks, W. W. Carotenoids: A Source of Flavor and Aroma. In *Biogeneration of Aromas*; H. P. Thomas and C. Rodney, Eds.; American Chemical Society: Washington, D.C., 1986; pp 157-166.

34 Winterhalter, P.; Rouseff, R. Carotenoid-Derived Aroma Compounds: an Introduction. In *Carotenoid-Derived Aroma Compounds*; P. Winterhalter and R. Rouseff, Eds.; American Chemical Society: Washington, DC, 2002; pp 1-17.

35 Knapp, H.; Straubinger, M.; Stingl, C.; Winterhalter, P. Analysis of Norisoprenoid Aroma Precursors. In *Carotenoid-Derived Aroma Compounds*; P. Winterhalter and R. L. Rouseff, Eds.; American Chemical Society: Washington, DC, 2002; pp 20-35.

36 Baumes, R.; Wirth, J.; Bureau, S.; Gunata, Y.; Razungles, A. Biogeneration of C13-norisoprenoid compounds: experiments supportive for an apo-carotenoid pathway in grapevines. *Anal. Chim. Acta* **2002**, *458*, 3-14.

37 Gunata, Z.; Dugelay, I.; Sapis, J. C.; Baumes, R.; Bayonove, C. Role of enzymes in the use of the flavor potential from grape glycosides in winemaking. *Prog. Flavour Precursor Stud. Proc. Int. Conf.* **1993**, 219-234.

38 Winterhalter, P. Oxygenated C13-Norisoprenoids: Important Flavor Precursors. In *Flavor Precursors: Thermal and Enzymatic Conversions*; R. Teranishi; G. R. Takeoka and M. Guntert, Eds.; American Chemical society: Washington, DC, 1992; pp 98-115.

39 Lutz, A.; Winterhalter, P. Isolation of additional carotenoid metabolites from quince fruit (*Cydonia oblonga* Mill.). *J. Agric. Food Chem.* **1992**, *40*, 1116-1120.

40 Aubert, C.; Ambid, C.; Baumes, R.; Guenata, Z. Investigation of Bound Aroma Constituents of Yellow-Fleshed Nectarines (Prunus persica L. Cv. Springbright). Changes in Bound Aroma Profile during Maturation. *J. Agric. Food Chem.* **2003**, *51*, 6280-6286.

41 Buttery, R. G.; Teranishi, R.; Ling, L. C.; Flath, R. A.; Stern, D. J. Quantitative studies on origins of fresh tomato aroma volatiles. *J. Agric. Food Chem.* **1988**, *36*, 1247-1250.

42 Wilkins, C. K. Paprika: relationships between aroma profile data and both GC and HPLC data. *Lebensm-Wiss.-Technol.* **1992**, *25*, 212-218.

43 Enzell, C. R.; Wahlberg, I. Tobacco isoprenoids - precursors of important aroma constituents. *Pure Appl. Chem.* **1990**, *62*, 1353-1356.

44 Kawakami, M.; Ganguly, S. N.; Banerjee, J.; Kobayashi, A. Aroma composition of oolong tea and black tea by brewed extraction method and characterizing compounds of darjeeling tea aroma. *J. Agric. Food Chem.* **1995**, *43*, 200-207.

45 Suzuki, M.; Matsumoto, S.; Fleischmann, H.-P.; Shimada, H.; Yamano, Y.; Ito, M.; Watanabe, N. Identification of β-damascenone Progenitors and Their Biogenesis in Rose Flowers (*Rosa damascena* Mill.). In *Carotenoid-Derived Aroma Compounds*; P. Winterhalter and R. L. Rouseff, Eds.; American Chemical Society: Washington, DC, 2002; pp 89-101.

46 Kotseridis, Y.; Baumes, R. L.; Skouroumounis, G. K. Quantitative determination of free and hydrolytically liberated .beta.-damascenone in red grapes and wines using a stable isotope dilution assay. *J. Chromatogr., A* **1999**, *849*, 245-254.

47 Sefton, M. A.; Francis, I. L.; Williams, P. J. Volatile norisoprenoid compounds as constituents of oak woods used in wine and spirit maturation. *J. Agric. Food Chem.* **1990**, *38*, 2045-2049.

48 Ohloff, G. Importance of minor components in flavors and fragrances. *Perfum. Flavor.* **1978**, *3*, 11-12, 14-16, 18-22.

49 Roberts, D. D.; Mordehai, A. P.; Acree, T. E. Detection and Partial Characterization of Eight β-Damascenone Precursors in Apples (Malus domestica Borkh. Cv. Empire). *J. Agric. Food Chem.* **1994**, *42*, 345-349.

50 Buttery, R. G.; Teranishi, R.; Ling, L. C.; Turnbaugh, J. G. Quantitative and sensory studies on tomato paste volatiles. *J. Agric. Food Chem.* **1990**, *38*, 336-340.

51 Buttery, R. G.; Ling, L. C. Volatile Components of Tomato Fruit and Plant Parts: Relationship and Biogenesis. In *Bioactive Volatile Compounds from Plants*; R. Teranishi; R. G. Buttery and H. Sugisawa, Eds.; American Chemical Society: Washington, DC, 1993; pp 23-34.

52 Lozano, P.; Delgado, D.; Gomez, D.; Rubio, M.; Iborra, J. L. A non-destructive method to determine the safranal content of saffron (Crocus sativus L.) by supercritical carbon dioxide extraction combined with high-performance liquid chromatography and gas chromatography. *J. Biochem. Biophys. Methods* **2000**, *43*, 367-378.

53 Knapp, H.; Straubinger, M.; Witte, A.; Winterhalter, P. Aroma Formation in Saffron. In *Frontiers of Flavour Science*; P. Winterhalter, Ed.: Freising, Germany, 2000; pp 440-444.

54 Razungles, A.; Bayonove, C. L.; Cordonnier, R. E.; Sapis, J. C. Grape carotenoids: changes during the maturation period and localization in mature berries. *Am. J. Enol. Vitic.* **1988**, *39*, 44-48.

55 Razungles, A.; Gunata, Z.; Pinatel, S.; Baumes, R.; Bayonove, C. Content of terpenes, norisoprenoids and their precursors in several varieties of grapes. *Sci. Aliments* **1993**, *13*, 59-72.

56 Schreier, P.; Drawert, F.; Junker, A. Identification of volatile constituents from grapes. *J. Agric. Food Chem.* **1976**, *24*, 331-336.

57 Williams, P. J.; Sefton, M. A.; Wilson, B. Nonvolatile Conjugates of Secondary Metabolites as Precursors of Varietal Grape Flavor Components. In *Flavor Chemistry: Trends and Development*; R. Teranishi; R. G. Buttery and F. Shahidi, Eds.; American Chemical Society: Washington, DC, 1989; pp 35-48.

58 Schneider, R.; Razungles, A.; Augier, C.; Baumes, R. Monoterpenic and norisoprenoidic glycoconjugates of Vitis vinifera L. cv. Melon B. as precursors of odorants in Muscadet wines. *J. Chromatogr., A* **2001**, *936*, 145-157.

59 Acree, T. E.; Barnard, J.; Cunningham, D. G. A procedure for the sensory analysis of gas chromatographic effluents. *Food Chem.* **1984**, *14*, 273-286.

60 Schieberle, P.; Grosch, W. Identificierung von Aromastoffen ausder Kruste von Roggenbrot - Verleich mit den Aromastoffen der Krume. *Z. Lebensm.-Unters. Forsch.* **1984**, 479-483.

61 da Silva, M. A. A. P.; Lundahl, D. S.; M.R., M. The Capabilities and Psychophysics of Osme: a New GC-Olfactometry Technique. In *Trends in Flavor Research*; H. Maarse and V. D. Heij, Eds.; Elsevier: New York, 1994; pp 191-210.

62 Stevens, S. S. Concerning the psychophysical power law. *Quart. J. Exp. Psychol.* **1964**, *16*, 383-385.

63 Arthur, C. L.; Pawliszyn, J. Solid phase microextraction with thermal desorption using fused silica optical fibers. *Analytical Chemistry* **1990**, *62*, 2145-2148.

64 Jia, M.; Zhang, Q. H.; Min, D. B. Optimization of Solid-Phase Microextraction Analysis for Headspace Flavor Compounds of Orange Juice. *J. Agric. Food Chem.* **1998**, *46*, 2744-2747.

65 Bezman, Y.; Rouseff, R. L.; Naim, M. 2-Methyl-3-furanthiol and methional are possible off-flavors in stored orange juice: aroma-similarity, NIF/SNIF GC-O, and GC analyses. *J. Agric. Food Chem.* **2001**, *49*, 5425-5432.

66 Steffen, A.; Pawliszyn, J. Analysis of Flavor Volatiles Using Headspace Solid-Phase Microextraction. *J. Agric. Food Chem.* **1996**, *44*, 2187-2193.

67 Song, J.; Fan, L.; Beaudry, R. M. Application of Solid Phase Microextraction and Gas Chromatography/Time-of-Flight Mass Spectrometry for Rapid Analysis of Flavor Volatiles in Tomato and Strawberry Fruits. *J. Agric. Food Chem.* **1998**, *46*, 3721-3726.

68 Rouseff, R.; Raley, L.; Hofsommer, H.-J. Application of diode array detection with a C-30 reversed phase column for the separation and identification of saponified orange juice carotenoids. *J. Agric. Food Chem.* **1996**, *44*, 2176-2181.

69 Winterhalter, P.; Lutz, A.; Schreier, P. Isolation of a glucosidic precursor of isomeric marmelo lactones from quince fruit. *Tetrahedron Lett.* **1991**, *32*, 3669-3670.

70 Lutz, A.; Winterhalter, P. Bio-oxidative cleavage of carotenoids: important route to physiological active plant constituents. *Tetrahedron Lett.* **1992**, *33*, 5169-5172.

71 Kotseridis, Y.; Baumes, R. L.; Bertrand, A.; Skouroumounis, G. K. Quantitative determination of b-ionone in red wines and grapes of bordeaux using a stable isotope dilution assay. *Journal of Chromatography, A* **1999**, *848*, 317-325.

72 Lee, H. S.; Castle, W. S. Seasonal Changes of Carotenoid Pigments and Color in Hamlin, Early gold, and Budd Blood Orange Juices. *J. Agric. Food Chem.* **2001**, *49*, 877-882.

73 Pupin, A. M.; Dennis, M. J.; Toledo, M. C. F. HPLC analysis of carotenoids in orange juice. *Food Chem.* **1999**, *64*, 269-275.

74 Philip, T.; Chen, T. S.; Nelson, D. B. Liquid chromatographic profiles of major carotenoid esters in commercially processed California Navel and Valencia orange juice concentrates. *J. Chromatogr.* **1988**, *442*, 249-265.

75 Lee, H. S.; Castle, W. S.; Coates, G. A. High-performance liquid chromatography for the characterization of carotenoids in the new sweet orange (Earlygold) grown in Florida, USA. *J. Chromatogr., A* **2001**, *913*, 371-377.

76 Mouly, P. P.; Gaydou, E. M.; Lapierre, L.; Corsetti, J. Differentiation of several geographical origins in single-strength Valencia orange juices using quantitative comparison of carotenoid profiles. *J. Agric. Food Chem.* **1999**, *47*, 4038-4045.

77 Noga, G.; Lenz, F. Separation of citrus carotenoids by reversed-phase high-performance liquid chromatography. *Chromatographia* **1983**, *17*, 139-142.

78 Marais, J. 1,1,6-Trimethyl-1,2-dihydronaphthalene (TDN): a possible degradation product of lutein and b-carotene. *South African J. Enol. Viticulture* **1992**, *13*, 52-55.

79 Chen, C. S.; Shaw, P. E.; Parish, M. E. Orange and Tangerine Juice. In *Fruit Juice Processing Technology*; S. Nagy; C. S. Chen and P. E. Shaw, Eds.; AGScience, Inc.: Auburndale, 1993; pp 110-165.

80 Stewart, I.; Wheaton, T. A. Continuous flow separation of carotenoids by liquid chromatography. *J. Chromatogr.* **1971**, *55*, 325-336.

81 Britton, G. UV/Visible Spectroscopy. In *Carotenoids, Vol. 1A: Isolation and Analysis*; G. Britton; S. Liaaen-Jenson and H. Pfander, Eds.; Birkhauser Verlag: Basel, Boston, Berlin, 1995; pp 13-62.

82 De Ritter, E.; Purcell, A. E. Carotenoid analytical Methods. In *Carotenoids as Colorants and Vitamin A Precursors*; J. C. Bauernfeind, Ed.; Academic Press: London, 1981; pp 815-923.

83 Farin, D.; Ikan, R.; Gross, J. The carotenoid pigments in the juice and flavedo of a mandarin hybrid (*Citrus reticulata*) cv. Michal during ripening. *Phytochemistry* **1983**, *22*, 403-408.

84 Curl, A. L.; Bailey, G. F. The carotenoids of Navel oranges. *J. Food Sci.* **1961**, *26*, 442-447.

85 Robertson, G. W.; Griffiths, D. W.; Woodford, J. A. T.; Birch, A. N. E. Changes in the chemical composition of volatiles released by the flowers and fruits of the red raspberry (*Rubus idaeus*) cultivar glen Prosen. *Phytochemistry* **1995**, *38*, 1175-1179.

86 Castellar, M. R.; Montijano, H.; Manjon, A.; Iborra, J. L. Preparative high-performance liquid chromatographic purification of saffron secondary metabolites. *J. Chromatogr.* **1993**, *648*, 187-190.

87 Gross, J.; Gabai, M.; Lifshitz, A. Carotenoids in juice of Shamouti orange. *J. Food Sci.* **1971**, *36*, 466-473.

88 Valim, M. F.; Rouseff, R. L.; Lin, J. Gas Chromatographic-Olfactometric Characterization of Aroma Compounds in Two Types of Cashew Apple Nectar. *J. Agric. Food Chem.* **2003**, *51*, 1010-1015.

89 Ahmed, E. M.; Dennison, R. A.; Shaw, P. E. Effect of selected oil and essence volatile components on flavor quality of pumpout orange juice. *J. Agric. Food Chem.* **1978**, *26*, 368-372.

90 Poole, C. F. *The essence of chromatography*; Elsevier: Amsterdam, Boston, London, New York, Oxford, Paris, san Diego, San Francisco, Singapore, Sidney, Tokyo, 2003.

91 Zhang, Z.; Pawliszyn, J. Headspace solid-phase microextraction. *Anal. Chem.* **1993**, *65*, 1843-1852.

92 Bao, M.; Mascini, M.; Griffini, O.; Burrini, D.; Santianni, D.; Barbieri, K. Headspace solid-phase microextraction for the determination of trace levels of taste and odor compounds in water samples. *Analyst (Cambridge, United Kingdom)* **1999**, *124*, 459-466.

93 Radford, T.; Kawashima, K.; Friedel, P. K.; Pope, L. E.; Gianturco, M. A. Distribution of volatile compounds between the pulp and serum of some fruit juices. *J. Agric. Food Chem.* **1974**, *22*, 1066-1070.

94 Lin, J.; Rouseff, R. L.; Barros, S.; Naim, M. Aroma Composition Changes in Early Season Grapefruit Juice Produced from Thermal Concentration. *J. Agric. Food Chem.* **2002**, *50*, 813-819.

95 Sanderson, G. W.; Grahamm, H. N. Formation of black tea aroma. *J. Agric. Food Chem.* **1973**, *21*, 576-585.

96 Day, W. C.; Erdman, J. G. Ionene; a thermal degradation product of β-carotene. *Science* **1963**, *141*, 808.

97 Mader, I. β-Carotene: thermal degradation. *Science* **1964**, *144*, 533-534.

98 Schiedt, K.; Liaaen-Jensen, S. Isolation and Analysis. In *Carotenoids*; G. Britton; S. Liaaen-Jensen and H. Pfander, Eds.; Birkhauser Verlag: Basel, Boston, Berlin, 1995; pp 81-108.

99 Ravichandran, R. Carotenoid composition, distribution and degradation to flavor volatiles during black tea manufacture and the effect of carotenoid supplementation on tea quality and aroma. *Food Chem.* **2002**, *78*, 23-28.

100 Stevens, M. A. Relationship Between Polyene-Carotene Content and Volatile Compound Composition of tomatoes. *J. Amer. Soc. Hort. Sci.* **1970**, *95*, 461-464.

101 Mordi, R. C.; Walton, J. C.; Burton, G. W.; Hughes, L.; Ingold, K. U.; Lindsay, D. A. Exploratory study of β-carotene autoxidation. *Tetrahedron Lett.* **1991**, *32*, 4203-4206.

102 de Heij, H. T.; van Dort, H. M. Autoxidation of carotenes. *Prog. Flavour Precursor Stud. Proc. Int. Conf.* **1993**, 409-413.

BIOGRAPHICAL SKETCH

Kanjana Mahattanatawee was born in Bangkok, Thailand on 17 May, 1965. She received a B.Sc. in biology with a major in Microbiology in 1988 from Sri-Nakharinwirot University, Thailand. She continued to pursue her Master of Science degree in the area of industrial microbiology at the Department of Microbiology, Chulalongkorn University, Bangkok Thailand from 1988-1991. From 1991-1992 she worked as a researcher, in the Department of Microbiology, Chulalongkorn University, Thailand. In 1992-1993 Kanjana was awarded a UNESCO scholarship to earn her Diploma in Microbiology and Biotechnology from Osaka University, Japan. Kanjana was appointed to a position as Lecturer, Department of Food Technology, Faculty of Science, Siam University from 1993-1999. From 1995-1997 she was an adjunct lecturer, Faculty of Environment and Natural Resource, Mahidol University. Kanjana conducted research and taught two microbiology courses (Industrial Microbiology and Fermentation Technology) for undergraduate students at the Faculty of Science, Siam University, Bangkok, Thailand.

She was awarded a scholarship from Siam University to pursue her Ph.D. In Spring 1999, she enrolled in the graduate program at the Department of Food Science and Human Nutrition at the University of Florida under Dr. R.L. Rouseff's supervision. She considers herself very fortunate to be enrolled in one of the greatest graduate programs in flavor chemistry, with excellent scientists who are a pleasure to work with. She completed her research for her Ph.D. degree at the Citrus Research and Education Center (CREC) in Lake Alfred, Florida.

After completing her Ph.D. program, Kanjana plans to work as a postdoctoral researcher to gain more experience in this subject area. Later, she will return to Thailand to fulfill an appointed position as an associate professor at Siam University. Kanjana will teach and conduct research. She hopes to deliver the excitement and enthusiasm to her students in Thailand that she has experienced from her professors in the U.S.A.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Russell L. Rouseff, Chair
Professor of Food Science and Human Nutrition

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Maurice R. Marshall, Jr.
Professor of Food Science and Human Nutrition

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Renee M. Goodrich
Assistant Professor of Food Science and Human Nutrition

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

David H. Powell
Faculty Scientist of Chemistry

This dissertation was submitted to the Graduate Faculty of the College of Education and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

May 2004

Dean, College of Agriculture and Life Science

Dean, Graduate School